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(54) Title: GPIbα FRAGMENTS AND RECOMBINANT DNA EXPRESSION VECTORS

(57) Abstract

Peptides and other polymers which inhibit the binding of von Willebrand factor to platelet membrane glycoprotein Ib and/or glycoprotein Ib expressed on the surface of any cell of megakaryocytic lineage and methods of inhibiting platelet activation, adhesion of platelets to surfaces, platelet aggregation, or thrombosis. Also, recombinant DNA expression vectors encoding a peptide which inhibits binding of von Willebrand factor to platelet membrane glycoprotein Ib, said vector including a nucleotide sequence encoding the amino acid sequence from His^{1} to Ala^{302}, inclusive, of the amino terminal region of platelet membrane glycoprotein Iba, or any sequential subset thereof; mammalian host cells transformed by said vectors; and a process for producing a peptide having the identifying characteristics of the 45 kDa tryptic fragment of glycocalicin and a process for expressing the full length GPIBa polypeptide (His^{1}-Leu^{610}) or a subfragment thereof.

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GPIbα FRAGMENTS AND RECOMBINANT DNA EXPRESSION VECTORS

CROSS-REFERENCE TO RELATED APPLICATIONS

This is a continuation-in-part of copending application Serial No. 07/613,083, filed November 14, 1990, which is a continuation-in-part of Serial No. 07/460,674, filed on January 4, 1990, which is a continuation-in-part of Serial No. 07/121,454, filed on November 17, 1987.

The invention described and claimed in the aforementioned '674 application relates to a class of peptides useful for inhibiting the binding of von Willebrand factor (vWF) to platelet membrane glycoprotein Ib (GPIb). The present application is concerned with the subject matter of the '674 application and also with novel DNA expression vectors encoding peptides and polypeptides useful for inhibiting the binding of vWF to GPIb, such peptides and polypeptides including the types referred to in the '674 application.

FIELD OF THE INVENTION

This invention relates to (A) peptides and polypeptides which inhibit the binding of von Willebrand factor to platelet membrane glycoprotein Ib and GPIb expressed on the surface of any cell of megakaryocytic lineage; (B) the use of these peptides and polypeptides in the prevention of platelet

activation, aggregation and surface adhesion; and (C) to the use of these peptides and polypeptides in the prevention of thrombosis. The present invention relates also to (D) recombinant DNA expression vectors which encode peptides and polypeptides which inhibit binding of vWF to GPIb, wherein said peptides and polypeptides include the amino terminal region of platelet membrane glycoprotein Ibα (GPIbα), or any sequential subset thereof; and (E) host cells transformed by such vectors. These vectors are useful in the production of peptides and polypeptides which can be used, for example, in the prevention of platelet activation, aggregation and surface adhesion, and also in the prevention of thrombosis.

When conditions such as, for example, trauma, surgery or disease disrupt the vascular endothelial lining, thereby exposing the subendothelial connective tissue to blood, the initial hemostatic response is platelet plug formation, also known as "primary hemostasis." One of the critical events in this process is the adhesion of platelets to the exposed subendothelial tissue. vWF mediates this adhesion by binding both to the GPIb receptor found on the surface of the platelet membrane and also to the subendothelial collagen fibrils found in the vascular subendothelium. This action by vWF enables platelet adhesion to occur under the conditions of high shear stress often found in damaged or diseased tissue, as caused by, for example, high flow rate in small vessels. This is of critical importance in stopping blood loss from capillaries, small arterioles and venules.

The importance of the interaction between vWF and GPIb is suggested by the bleeding diathesis of Bernard-Soulier syndrome, a disorder characterized by decreased quantities or abnormal function of GPIb and, consequently, markedly reduced platelet adhesion due to the inability of vWF to bind with GPIb.

Inhibition of vWF-GPIb interaction would thus be expected to result in the prevention of primary hemostasis and the induction of an anti-thrombotic state useful in prevention of diseases in which occlusion of blood vessels plays an important role. The proteolytic fragments of GPIb and peptides and polypeptides produced using the vectors of the present invention have the ability to act as anti-thrombotic agents by their prevention of the binding of vWF to GPIb.

By way of background, it is noted that GPIb is a two-chain molecule having an apparent molecular mass of approximately 160 kDa. GPIb is composed of a heavy (alpha, or GPIbα) chain, having a molecular mass of approximately 145 kDa, linked by disulfide bonds to a light (beta, or GPIbβ) chain, having a molecular mass of approximately 22 kDa. GPIb is an integral membrane protein and both the alpha- and beta- chains described above have transmembrane domains. Proteolysis by an endogenous calcium-dependent platelet protease generates a proteolytic fragment from the amino-terminal portion of GPIbα, which is known as glycocalicin and which consists of nearly the entire GPIbα chain, having an approximate molecular mass of 140 kDa. This fragment originates from the extracellular domain of GPIbα and is water soluble. Thus, it is released after cleavage from the parent molecule.

A complete cDNA encoding human GPIbα polypeptide has been determined by Lopez et al., Proc. Natl. Acad. Sci. USA, **84**, 5615-5617 (1987), a publication which is not prior art. For convenience, the amino acid numbering system of Lopez et al., above, is followed herein. Also, the gene for GPIbα has been cloned from a genomic cosmid library utilizing a partial cDNA clone as a probe, and its sequence, including introns, has been determined by Wenger, Biochemical and Biophysical Research Communications, **156**(1), 389-395 (1988). The

nucleotide numbering system of Wenger, above, is followed herein.

The predicted GPIbα sequence consists of a 16 amino acid signal peptide, Met^{-16} through Pro^{-1}, followed by a 610 amino acid mature peptide or polypeptide region, His^{1} through Leu^{610}. As shown in Table I below, the complete sequence of the 45 kDa tryptic fragment comprises His^{1} through Arg^{290} or Arg^{293}. For the purposes of this application, as GPIbα and glycocalicin have the same amino terminus and are nearly identical in size, references to glycoalicin fragments and GPIbα fragments herein should be considered equivalent.

Trypsin has been previously shown to cleave glycocalicin between residues Arg^{290}/Ala^{291} and/or Arg^{293}/Thr^{294} to generate two fragments, one of which has an apparent molecular mass of 45 kDa and extends from the amino terminal residue His^{1} to Arg^{290} or Arg^{293}; the other, with an apparent molecular mass of 84 kDa, is very rich in carbohydrate and represents the carboxyl terminal half of glycocalicin beginning at Ala^{291} or Thr^{294}. The 45 kDa fragment consists of a single-chain species and a two-chain species. The latter is generated by an additional tryptic cleavage between residues Lys^{237} and Ala^{238} yielding two polypeptides of apparent molecular mass 35 kDa and 7 kDa, held together by one or more interchain disulfide bonds. The relative proportions of the one- and two-chain species depend on the extent of tryptic cleavage of glycocalicin. For example, after digestion for 18 hours with an enzyme to substrate ratio of 1:200 (w/w), the two-chain species predominates. The two chains of this species can be separated by reduction of the disulfide bonds and end-blocking of the resulting sulfhydryl groups, for example, by treatment with a molar excess of dithiothreitol and by S-carboxyimidomethylation with iodoacetamide, respectively.

SUMMARY OF THE INVENTION

As set forth in aforementioned application Serial No. 07/470,674, there are provided peptides comprising any peptide of the 45 kDa amino terminal tryptic fragment of glycocalicin selected from the amino acid sequence shown below in Table I:

Table I

```
          10        20        30        40        50        60
          |         |         |         |         |         |
HPICEVSKVASHLEVNCDKRNLTALPPDLPKDTTILHLSENLLYTFSLATLMPYTRLTQL

          70        80        90        100       110       120
          |         |         |         |         |         |
NLDRCELTKLQVDGTLPVLGTLDLSHNQLQSLPLLGQTLPALTVLDVSFNRLTSLPLGAL

          130       140       150       160       170       180
          |         |         |         |         |         |
RGLGELQELYLKGNELKTLPPGLLTPTPKLEKLSLANNNLTELPAGLLNGLENLDTLLLQ

          190       200       210       220       230       240
          |         |         |         |         |         |
ENSLYTIPKGFFGSHLLPFAFLHGNPWLCNCEILYFRRWLQDNAENVYVWKQGVDVKAMT

          250       260       270       280       290
          |         |         |         |         |
SNVASVQCDNSDKFPVYKYPGKGCPTLGDEGDTDLYDYYPEEDTEGDKVRATR
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and which inhibit the binding of von Willebrand factor to platelet membrane glycoprotein Ib and/or GPIb expressed on the surface of any cell of megakaryocytic lineage.

The invention further comprises any sequential subset of the 45 kDa amino terminal tryptic fragment of glycocalicin selected from the foregoing amino acid sequence which inhibits binding of vWF to GPIb and/or GPIb expressed on the surface of any cell of megakaryocytic lineage.

In addition, the invention comprises a peptide which inhibits binding of vWF to GPIb and/or GPIb expressed on the

surface of any cell of megakaryocytic lineage selected from the group of peptides consisting of:

DKRNLTALPPDLPKDTT; NLTALPPDLPKDTTI; PPDLPKDTTILHLSE; PGLLTPTPKLEKLSL; KQGVDVKAMTSNVAS; GDTDLYDYYPEEDTE; EEDTEGDKVRATRTV; PPDLPKDTT; and EEDTE.

An additionally preferred peptide is a peptide of any sequential subset of amino acids of a peptide which inhibits binding of vWF to GPIb and/or GPIb expressed on the surface of any cell of megakaryocytic lineage.

The invention further comprises peptides having the general formula $(KR)_n$, wherein n=2-10 or R_n, wherein n=2-20 and any derivatives thereof which inhibit binding of vWF to GPIb and/or GPIb expressed on the surface of any cell of megakaryocytic lineage.

The invention comprises also a method for inhibiting activation of platelets, adhesion of platelets to surfaces, or aggregation of platelets to each other with an effective amount of one of the aforementioned peptides or subsets, or other polymers as described therein.

Another aspect of the invention comprises a method for inhibiting thrombosis in a patient which comprises administering to said patient an effective amount of one of the aforementioned peptides or subsets, or other polymers as described therein.

As also set forth in the aforementioned '674 parent application, a complete cDNA encoding human GPIbα polypeptide has been determined by Lopez et al. With such information, a nucleotide sequence can be inserted into an appropriate vector for the expression of peptides from the 45 kDa fragment. Accordingly, another aspect of the invention is the provision

of a recombinant DNA expression vector encoding a peptide or polypeptide which inhibits binding of vWF to GPIb, said vector including a nucleotide sequence encoding the amino acid sequence from HIS^{1} to LEU^{610}, inclusive, of the amino terminal region of GPIbα, or any sequential subset thereof. Particularly preferred are those vectors including a nucleotide sequence which endcodes a peptide including the amino acid sequence from HIS^{1} to THR^{294}, inclusive, of GPIbα.

Still another aspect of the invention relates to a recombinant DNA expression vector encoding a peptide which includes, and extends beyond, at the carboxyl terminal region, the 45 kDa tryptic fragment of glycocalicin, and which, upon expression in a suitable transformed host cell, produces a peptide having the biological activity of the aforementioned 45 kDa fragment.

The invention relates also to a host cell which has been transformed by any the aforementioned vectors. Particularly preferred host cells include mammalian host cells.

Another aspect of the invention relates to a process for producing peptides or polypeptides having the biological activity of the 45 kDa tryptic fragment of glycocalicin comprising maintaining any of the aforementioned transformed host cells under conditions permitting the expression of the peptide.

An additional aspect of the invention provides a process for expressing the full length GPIbα polypeptide (His^{1}-Leu^{610}) or a subfragment thereof which involves constructing a DNA sequence which encodes the full length polypeptide, inserting said DNA sequence into a suitable plasmid or vector, transforming a host cell with said modified plasmid or vector and maintaining the transformed host cell under conditions

which result in expression within the host cell of the full length polypeptide or a fragment thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph which shows the inhibitory effect of peptide subfragments of the 45 kDa amino terminal region of GPIbα on ristocetin-dependent and botrocetin-dependent vWF binding to platelet GPIb receptors.

Figure 2 is a pair of graphs which show the inhibitory effect of the GPIbα peptide fragment consisting of residue positions 271 to 285 on (A) botrocetin-dependent and (B) ristocetin-dependent vWF binding to platelet GPIb receptors.

Figure 3 is a graph which shows the inhibitory effects of the GPIbα peptide fragment consisting of residue positions 251 through 279 on botrocetin-dependent and ristocetin-dependent vWF binding to platelet GPIb receptors.

Figure 4 is a dot blot profile demonstrating the reactivity of GPIbα polypeptides, produced by pMW1 and pMW2 transformed cells, to conformation dependent anti-GPIbα monoclonal antibodies.

Figure 5 is an immunoblot showing intracellular processing of the pMW2 polypeptide in host cells.

Figure 6 is a graph which shows that the GPIbα antigen produced by pMW2 is functionally active in a botrocetin-induced binding assay.

DETAILED DESCRIPTION OF THE INVENTION

For purposes of this disclosure, accepted short-hand designations of the amino acids have been used. The designations are shown in Table II below.

Table II

One and three-letter Amino Acid abbreviations

A	ALA	Alanine
C	CYS	Cysteine
D	ASP	Aspartic Acid
E	GLU	Glutamic Acid
F	PHE	Phenylalanine
G	GLY	Glycine
H	HIS	Histidine
I	ILE	Isoleucine
K	LYS	Lysine
L	LEU	Leucine
M	MET	Methionine
N	ASN	Asparagine
P	PRO	Proline
Q	GLN	Glutamine
R	ARG	Arginine
S	SER	Serine
T	THR	Threonine
V	VAL	Valine
W	TRP	Tryptophan
Y	TYR	Tyrosine
B	ASX	Asp or Asn, not distinguished
Z	GLX	Glu or Gln, not distinguished
X	X	Undetermined or atypical amino acid

Definitions

Unless indicated otherwise herein, the following terms have the indicated meanings.

Codon - A DNA sequence of three nucleotides (a triplet) which encodes through mRNA an amino acid, a translation start signal or a translation termination signal. For example, the DNA nucleotide triplets TTA, TTG, CTT, CTC, CTA and CTG encode the amino acid leucine (LEU); TAG, TAA and TGA are translation

stop signals; and ATG is a translation start signal encoding methionine (MET).

Structural Gene - A DNA sequence which encodes through its corresponding messenger RNA (mRNA) a sequence of amino acids characteristic of a specific polypeptide. Structural genes may also have RNA as their primary product, for example, transfer RNA (tRNA) or ribosomal RNA (rRNA).

Transcription - The process of producing RNA from a structural gene.

Translation - The process of producing a polypeptide from mRNA.

Coding Sequence (Encoding DNA) - DNA sequences which, in the appropriate reading frame, code for the amino acids of a protein. For the purpose of the present invention, it should be understood that the synthesis or use of a coding sequence may necessarily involve synthesis or use of the corresponding complementary strand, as shown by: 5'-CGG·GGA·GGA-3', which has a complementary strand which is 3'-GCC·CCT·CCT-5', and which encodes the tripeptide NH_2-arg-gly-gly-CO_2H. A discussion of or claim to one strand is deemed to refer to or to claim the other strand and the double stranded counterpart thereof as is appropriate, useful or necessary in the practice of the art.

cDNA - A DNA molecule or sequence which has been enzymatically synthesized from the sequence(s) present in an mRNA template.

Transcribed Strand - The DNA strand whose nucleotide sequence is read 3' → 5' by RNA polymerase to produce mRNA. This strand is also referred to as the noncoding strand.

Non-Transcribed Strand - This strand is the antiparallel compliment of the transcribed strand and has a base sequence identical to that of the mRNA produced from the transcribed strand except that thymine bases are present (instead of uracil bases of the mRNA). It is referred to as "coding" because, like mRNA, and when examined 5' → 3', the codons for translation may be directly discerned. This strand is also referred to as the coding strand.

Expression - The process undergone by a structural gene to produce a product. In the case of a protein product, it is a combination of transcription and translation.

Recombinant DNA Molecule - A molecule consisting of segments of DNA from different genomes which have been joined end-to-end and have, or can be modified to have, the capacity to infect some host cell and be maintained therein.

Biological Activity - One or more functions, effects of, activities performed or caused by a molecule in a biological context (that is, in an organism or in an in vitro facsimile). A characteristic biological activity of the amino terminal region of GPIbα is the ability to bind to vWF, an activity which may be demonstrated in vitro, for example, by the aggregation of platelets in the presence of ristocetin.

Reducing Conditions - Refers to the presence of a "reducing" agent in a solution containing vWF, or polypeptides derived therefrom, which agent causes the disruption of disulfide bonds of the vWF. However, consistent with usage typical in the art, the reducing agent, such as, for example, dithiothreitol (DTT), causes a vWF disulfide bond to be broken by forming a disulfide bond between a vWF cysteine and the DTT with no net change in oxidation state of the involved sulfur atoms.

Phage or Bacteriophage - A bacterial virus, many of which consist of DNA sequences encapsulated in a protein envelope or coat (capsid).

Promoter - A DNA sequence upstream from a gene which promotes its transcription.

Plasmid - A nonchromosomal double-stranded DNA sequence comprising an intact replicon such that the plasmid is replicated in a host cell. When the plasmid is placed within a procaryotic or eucaryotic host cell, the characteristics of that cell may be changed (or transformed) as a result of the DNA of the plasmid. For example, a plasmid carrying the gene for tetracycline resistance (Tet^R) transforms a cell previously sensitive to tetracycline into one which is resistant to it. A cell transformed by a plasmid is called a "transformant."

Cloning - The process of obtaining a population of organisms, or DNA sequences or other macromolecules derived from one such organism or sequence by asexual reproduction or DNA replication.

Expression Plasmid - A plasmid into which has been inserted the DNA being cloned, such as the vWF structural gene. The DNA sequence inserted therein may also contain sequences which control the translation of mRNA resultant therefrom, and may contain restriction endonuclease sites which facilitate assembly of, and may facilitate further modification of, the expression plasmid. An expression plasmid is capable of directing, in a host cell, the expression therein of the encoded polypeptide and usually contains a transcription promoter upstream from the DNA sequence of the encoded structural gene. An expression plasmid may or may not become integrated into the host chromosomal DNA. For the purpose of

this invention, an integrated plasmid is nonetheless referred to as an expression plasmid.

Viral Expression Vector - A viral expression vector is similar to an expression plasmid except that the DNA may be packaged into a viral particle that can transfect cells through a natural biological process.

Downstream - A nucleotide of the transcribed strand of a structural gene is said to be downstream from another region of the gene if the nucleotide is normally read by RNA polymerase after the other region of the gene. The complimentary nucleotide of the nontranscribed strand, or the corresponding base pair within the double stranded form of the DNA, are also denominated downstream.

Additionally, and making reference to the direction of transcription and of translation within the structural gene, a restriction endonuclease sequence added upstream (or 5') to the gene means it is added before the sequence encoding the amino terminal end of the protein, while a modification created downstream (or 3') to the structural gene means that it is beyond the carboxy terminus-encoding region thereof.

Glycoprotein Ibα or GPIbα - It is understood that all references herein to Glycoprotein Ibα refer to human GPIbα.

Mature GPIbα - Refers to a polypeptide consisting of the amino acid sequence His^{1} to Leu^{610} which is typically found in platelets as a transmembrane protein. Additionally, when expressed in mammalian cells, mature GPIbα is usually glycosylated. Certain mutations or polymorphisms have been described with respect to the amino acid sequence encoded by the human glycoprotein Ibα gene. A threonine/methionine polymorphism is known at position 145 in the mature GPIbα

sequence. In addition, for example, a 13 amino acid sequence (Ser Glu Pro Ala Pro Ser Pro Thr Thr Pro Glu Pro Thr) of the carboxy terminal region of the mature polypeptide is repeated two-fold in approximately half the human population. With respect to those polymorphisms which have been or may yet be discovered, it is expected that most such encoded polypeptide sequences would also be effective in the practice of the invention.

Signal Peptide (Sequence) - A signal peptide is the sequence of amino acids in a newly translated polypeptide which signals translocation of the polypeptide across the membrane of the endoplasmic reticulum and into the secretory pathway of the cell. A signal peptide typically occurs at the beginning (amino terminus) of the protein and is 20-40 amino acids long with a stretch of approximately 5-15 hydrophobic amino acids in its center. Typically the signal sequence is proteolytically cleaved from the protein during, or soon after, the process of translocation into the endoplasmic reticulum. That portion of a gene or cDNA encoding a signal peptide may also be referred to as a signal sequence.

The terms "peptide" and "polypeptide" are used herein interchangeably.

In the course of work on the present invention, purified glycocalicin was used in tests to evaluate the effectiveness of compounds in inhibiting the binding of von Willebrand factor to intact platelets.

Purified glycocalicin has been produced by a two-step procedure based on 1) affinity chromatography using wheat germ agglutinin insolubilized onto Sepharose® beads; and 2) subsequent immunoaffinity chromatography using a monoclonal

antibody (LJ-P3) directed against glycocalicin and insolubilized onto Sepharose® beads.

Outdated platelet concentrates were used as starting material for the purification of glycocalicin. Plasma components were eliminated by sedimenting the platelets at 2,300 g for 25 minutes at room temperature (22-25°C), removing the supernatant, and resuspending the platelet pellet in a buffer composed of 10 mM Tris base and 150 mM NaCl, adjusted to pH 7.4 with HCl (Tris-buffered saline; TBS), and containing 2 mM EDTA. This procedure was repeated twice. After the first wash, the suspension was centrifuged at 600 g for 1 minute and the pellet containing most of the contaminating red cells was discarded before continuing with the washing procedure. After the last centrifugation, the platelets were resuspended in TBS containing 2 mM $CaCl_2$ and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). They were then disrupted by sonication (three pulses of 15 seconds each at approximately 100 watts, with the platelet suspension kept on ice). The suspension was then left for three hours at room temperature and for 16-18 hours at 4°C, always with continuous stirring. Following this, the particulate material in the suspension was removed by centrifugation at 100,000 g for 20 minutes at 12°C. The clear supernatant was applied to a column (2.6 cm in diameter and 11 cm high) of wheat germ agglutinin bound to Sepharose® beads activated with cyanogen bromide and equilibrated with TBS containing 1 mM EDTA, 0.1 mM PMSF, and 0.02% sodium azide. The column was washed with a volume of buffer corresponding to twice the volume of beads before eluting bound proteins with 100 mM N-acetyl glucosamine added to the same buffer. The whole procedure was performed at room temperature. The eluted material was immediately applied to a monoclonal antibody column (5 cm in diameter and 2.5 cm high) consisting of purified IgG bound to Sepharose® beads activated with cyanogen bromide. The monoclonal

antibody used, designated LJ-P3, is specific for the glycocalicin portion of GPIb; its preparation, characterization, and purification are described in Handa et al., J. Biol. Chem., **261**, 12579-12585 (1986). The column was equilibrated with a buffer composed of 100 mM Tris base, 500 mM $LiCl_2$, 1 mM EDTA, 0.1 mM PMSF, 0.02% sodium azide, adjusted to pH 7.4 with HCl. The column was washed with a volume of buffer corresponding to three times the volume of the beads. Bound glycocalicin was eluted with 70-80 mℓ of 50 mM diethylamine containing 1 mM EDTA and 0.1 mM PMSF. During this step, the flow rate through the column was regulated so that elution was complete in 20-25 minutes. The whole procedure was performed at room temperature. The eluted glycocalicin was collected in 6 g of glycine to neutralize the high pH of diethylamine. The purified material was dialyzed extensively against TBS, concentrated with Aquacide®, and again dialyzed with TBS. Purified glycocalicin was stored in aliquots at -70°C.

Purified glycocalicin was digested with trypsin pretreated with N-tosyl-L-phenylalanine chloromethylketone. The enzyme substrate ratio was 1:200 and the reaction was allowed to proceed for 16-18 hours at 37°C. At the end of the incubation, trypsin activity was inhibited with a two-fold molar excess of (p-amidinophenyl)methanesulfonyl fluoride. The 45 kDa fragment of glycocalicin generated by trypsin digestion was purified by gel permeation high performance liquid chromatography using one GF 450 and two GF 250 duPont Zorbax® columns (9.4 mm in diameter by 25 cm in length) mounted in series. The columns were equilibrated with 200 mM $(NH_4)_2HPO_4$, pH 7, and the flow rate was 1 mℓ/minute. The procedure was performed at room temperature. The 45 kDa fragment eluted as a sharp peak and was then collected, concentrated with Aquacide®, dialyzed extensively with TBS, and stored in aliquots at -70°C until used.

The purified glycocalicin has been used to demonstrate that this proteolytic fragment of GPIbα can inhibit the binding of vWF to intact platelets. The assay system is based on the use of ^{125}I-labeled vWF and fresh or formalin-fixed platelets; ristocetin was used to induce the binding of vWF to GPIb. After incubation for 30 minutes at 37°C, without stirring, separation of bound platelet from free vWF ligand was achieved by centrifugation through 20% sucrose in Tyrode buffer, followed by measurement of the bound radioactivity as described in Ruggeri et al., J. Clin. Invest., **72**, 1-12 (1983). Nonspecific binding was evaluated for selected points by measuring the binding in the presence of a 40-fold excess of unlabeled vWF. Binding isotherms were evaluated by Scatchard-type analysis to determine binding parameters (including the estimate of nonspecific binding) using the computer-assisted program "Ligand" as described in Munson, Methods Enzymol., **92**, 542-576 (1983).

Glycocalicin at final concentrations in excess of 1 mg/mℓ can block the binding of ^{125}I-labeled vWF to intact GPIb completely; the concentration necessary to inhibit 50% of the binding (denoted as the IC_{50} value) averaged 150 μg/mℓ for seven different glycocalicin preparations.

Subsequently, all the intra-chain disulfide bonds present in glycocalicin were reduced by treatment with a molar excess of dithiothreitol and the resulting sulfhydryl groups blocked by S-carboxyimidomethylation. The resulting reduced and alkylated glycocalicin was found to retain the property of blocking vWF binding to intact GPIb on platelets in the presence of ristocetin. Since the reduced and alkylated glycocalicin had lost its secondary structure dependent on intra-chain disulfide bonds, this experiment demonstrated that the function of interacting with vWF could be ascribed to specific regions within the primary structure of glycocalicin.

The 45 kDa tryptic fragment of glycocalicin was purified using high performance liquid chromatography (abbreviated HPLC) and gel permeation columns that separate proteins on the basis of their molecular mass. Because of the conditions used for tryptic digestion, the 45 kDa fragment consisted essentially of the two-chain species. This purified proteolytic fragment of glycocalicin was used to test its ability to block the binding of vWF to the GPIb of platelets. The 45 kDa fragment inhibited completely the ristocetin-mediated binding of vWF to platelets, i.e. to GPIb, with an IC_{50} of approximately 3.5 μM.

In a similar experiment, glycocalicin was digested with trypsin, the disulfide bonds were reduced with dithiothreitol and the resulting sulfhydryl groups S-carboxyimidomethylated with iodoacetamide. The 35 kDa amino terminal fragment was then purified by gel permeation HPLC and tested for its inhibitory effect on the binding of vWF to the GPIb of platelets in a ristocetin-mediated assay. Its IC_{50} was found to be similar to that of the parent unreduced 45 kDa fragment. In accordance with the results obtained with whole glycocalicin, these results confirm that the primary structure of the amino terminal region of glycocalicin contains certain vWF binding domain(s) whose function does not depend on maintenance of the native three dimensional conformation of the molecule.

Following these findings, overlapping peptides of 15 amino acid residues each and representing the sequence of the entire 45 kDa amino terminal fragment of glycocalicin were synthesized. The following peptides were found to inhibit the binding of vWF to the GPIb of platelets with IC_{50} values of 0.5 mM or better (single-letter notation is used for the identification of amino acid residues): DKRNLTALPPDLPKDTT; NLTALPPDLPKDTTI; PPDLPKDTTILHLSE (these three peptides overlap

each other and cover the sequence between residues ASP^{18} and GLU^{40}, inclusive, of glycocalicin); PGLLTPTPKLEKLSL (residues PRO^{141} to LEU^{155}); KQGVDVKAMTSNVAS (residues LYS^{231} to SER^{245}); GDTDLYDYYPEEDTE; EEDTEGDKVRATRTV (these two peptides cover the sequence between residues GLY^{271} and VAL^{295}, inclusive). The results, therefore, clearly indicate the existence of multiple domains within the amino terminal region of glycocalicin that have functional relevance for vWF binding.

Shorter peptides with sequences corresponding to overlapping regions of the longer peptides exhibiting inhibitory activity were also synthesized. Two of these shorter peptides were found to have inhibitory activity. Their sequences were PPDLPKDTT (residues PRO^{26} to THR^{34} of glycocalicin) and EEDTE (residues GLU^{281} to GLU^{285}). These two peptides, when tested individually, had IC_{50} values greater than 0.5 mM. When they were combined together at a concentration of 0.5 mM, however, they completely inhibited vWF binding to GPIb. This experiment demonstrates that different noncontiguous domains within the primary sequence of glycocalicin may co-participate synergistically in providing the vWF binding site(s) and, consequently, the vWF binding activity. The sequence of the 45 kDa amino terminal tryptic fragment of glycocalicin, therefore, as well as subsets of it (as shown above) contains information useful for designing molecules capable of inhibiting the binding of vWF to the GPIb of platelets.

Peptides of the general formula $(KR)_n$, where n=2-7, and the peptide R_{11} also inhibit the interaction of vWF with GPIb. Peptides of the general formula R_nRGDV or $(KR)_nRGDV$ were previously demonstrated to block fibrinogen binding to GPIIb/IIIa (U.S. Patent No. 4,683,291, "Platelet Binding Inhibitors"). The latter peptides are now shown to be as

effective in blocking vWF-GPIb interaction as their $(KR)_n$ analogs and therefore represent a class of bifunctional antiplatelet agents.

The mechanisms responsible for triggering the binding of vWF to GPIb *in vivo* have not yet been determined. Normally, vWF and GPIb coexist in circulation without any significant interaction occurring. Contact with exposed or damaged subendothelium triggers binding and, possibly, vWF assumes an altered conformation (which is capable of complex formation) when contacting a blood vessel wall. See Sakariassen et al., Nature, **279**, 636-638 (1979); Stel et al., Blood, **65**, 85-90 (1985); and Turitto et al., Blood, **65**, 823-831 (1985). Conformational changes necessary for binding may also be induced in GPIb by contact of the platelet with other blood components or exposure of the platelet to high sheer stress in a damaged vessel. Moake et al., Blood, **71**, 1366-1374 (1988).

The interaction between vWF and GPIb can be demonstrated *in vitro* by several methods. Binding can be demonstrated in the presence of ristocetin, a glycopeptide antibiotic which may act by reducing excess negative charge density between the macromolecules. See Howard et al., Thromb. Diath. Haemorrh., **26**, 362-369 (1971); and Coller et al., J. Clin. Invest., **60**, 302-312 (1977). The interaction may also be triggered by the presence of the protein botrocetin, a component of certain snake venoms. Read et al., Proc. Nat'l. Acad. Sci. USA., **75**, 4514-4518 (1978). The interaction between vWF and GPIb can also be enhanced by removing terminal (negatively charged) sialic acid carbohydrate residues from the vWF molecule. De Marco et al., J. Clin. Invest., **68**, 321-328 (1981).

The *in vivo* relevance of the known standard *in vitro* binding assays, and binding inhibition assays, is not yet known. A detailed investigation of the mechanisms of *in vitro*

binding may eventually identify the in vivo mechanisms or important features thereof. The binding of vWF to GPIb as measured under the different experimental systems may also involve different functional domains of the macromolecules or conformational states thereof. Consequently, it is reasoned that those peptides derived from GPIb that would be particularly useful as therapeutic inhibitors of vWF binding, in vivo, would be those peptides which demonstrate significant inhibition of vWF binding in more than one in vitro assay system.

In addition, it is reasoned that the domains of GPIbα, and in particular the domains of the 45 kDa amino terminal fragment thereof which are responsible for binding vWF, are not necessarily adjacent to one another along the linear sequence of the amino terminal polypeptide, but that binding to vWF is accomplished by peptide sequences scattered throughout the 45 kDa polypeptide which are brought into proximal positions when the amino terminal fragment assumes its native tertiary structure.

Additional developmental work which forms the basis of the present application was conducted in light of the above hypotheses. As is demonstrated below, polymers are revealed which have increased vWF binding inhibition activity.

The present invention includes within its scope a synthetic peptide or polypeptide assembled from multiple native sequence fragments of the amino terminal portion of glycocalicin, which fragments are not proximal in the primary structure and which have a structure whose tertiary conformation displays binding domains which mimic the three dimensional binding domains of GPIb and which have a high affinity for vWF.

In addition, the present invention encompasses therapeutic polymers with multiple domains of amino acid sequences from GPIb which domains are connected by a linker which may or may not be of peptide character.

In the practice of the present invention, it is preferred to use the following peptides, identified in Table III, below, as inhibitors of the interaction of vWF with platelets.

Table III

	peptide	residue positions in glycocalicin
(a)	SDKFPVYKYPGKGCPTLGDEGDTDLYDYY	251-279
(b)	NLDRCELTKLQVDGT	61-75
(c)	QVDGTLPVLGTLDLS	71-85
(d)	TLDLSHNQLQSLPLL	81-95
(e)	QTLPALTVLDVSFNR	97-111
(f)	LKTLPPGLLTPTPKL	136-150
(g)	NCEILYFRRWLQDNA	210-224
(h)	QDNAENVYVWKQGVD	221-235
(i)	KQGVDVKAMTSNVAS	231-245
(j)	SNVASVQCDNSDKFP	241-255
(k)	SDKFPVYKYPGKGCP	251-265
(l)	GKGCPTLGDEGDTDL	261-275
(m)	GDTDLYDYYPEEDTE	271-285

Of the aforementioned peptides, the use of (a) or (m) is particularly preferred and the use of (a) is most preferred.

These peptides, as well as other polymers within the scope of the present invention, can be used individually or in combination with one or more other polymers of the present invention (whether or not covalently attached) in the inhibition of platelet activation, aggregation, or adherence to surfaces, or as a potential therapeutic anti-thrombotic.

The present invention includes also within its scope a peptide comprising any sequential subset of the amino acid

sequence of a peptide of (a) to (m) above and which inhibits binding of vWF to GPIb and/or GPIb expressed on the surface of any cell of megakaryocytic lineage.

Another aspect of the present invention encompasses a cysteine dimer of a peptide or other polymer within the scope of the present invention. Such dimers are compounds in which a cysteine residue of a peptide or polymer is covalently linked to a cysteine residue of another peptide or polymer by way of a disulfide bridge. A preferred cysteine dimer for use in the practice of the present invention is the dimer of the peptide SDKFPVYKYPGKGCPTLGDEGDTDLYDYY. It has been observed that under the <u>in vitro</u> conditions of vWF binding assays used in the present invention, the preferred dimer is particularly effective at inhibiting vWF binding.

Still another aspect of the present invention includes a polymer which inhibits binding of vWF to GPIb and/or GPIb expressed on the surface of any cell of megakaryocytic lineage and which includes the following domains:

domain A - a series of amino acids which constitutes any of the peptides of the present invention or of any subset of the amino acid sequence of said peptides;

domain B - a series of amino acids which constitutes any of the peptides of the present invention or of any subset of the amino acid sequence of said peptides and which may be the same or different from that of domain A; and

domain C - a linker which joins domain A and domain B.

The linker which joins segments of the aforementioned polymer can comprise monomeric or polymeric units assembled from units such as, for example, methylene, vinyl, amino acids and dextrans. A preferred polymer for use in the practice of

the present invention is one in which domain A comprises the peptide of (a) above and domain B comprises the peptide of (m) above. More preferably, there is used a polymer in which domain A comprises any subset of the amino acid sequence of the peptide of (a) above and domain B comprises any subset of the amino acid sequence of the peptide of (m) above.

It should be understood that polymers of the aforementioned types can include also one or more additional domains which impart desired functional properties to the polymer such as enhanced binding or solubility.

In addition, the present invention includes within its scope a synthetic polymer which inhibits binding of vWF to GPIb and/or GPIb expressed on the surface of any cell of megakaryocytic lineage and which comprises one or more sequences of amino acids of the GPIbα chain, said sequence(s) being normally positioned at or near the surface of the GPIbα chain in its native conformation and capable of interacting with vWF.

It should be understood that the present invention also includes within its scope derivatives of any of the peptides or other polymers of the present invention. Such derivatives include peptides or other polymers which have been modified by the addition of additional polymer sequence or by the addition of functional groups such as, for example, acetyl, glycosyl or ester moieties.

To carry out the assessments of this invention of the utility of numerous overlapping GPIbα peptides to inhibit binding of vWF to platelets, peptides based on the amino acid sequence of the 45 kDa amino terminal tryptic fragment of glycocalicin were synthesized as described by Houghton et al.,

Proc. Natl. Acad. Sci. USA, **82**, 5135 (1985). See also Vicente et al., J. Biol. Chem., **268**(34), 18473-18479 (1988).

In the well known procedure for solid-phase synthesis of a peptide, the desired peptide is assembled starting from an insoluble support such as benzhydryl amine or chloromethylated resin (derived from cross-linked polystyrene, and available from chemical supply houses). The amino acid at the carboxy-terminal end of the desired peptide, carrying protecting groups on the α-amino nitrogen and on any other reactive sites, is attached to the resin from solution using known peptide coupling techniques. The protecting group on the alpha-amino group is removed (leaving other protecting groups, if any, intact), and the next amino acid of the desired sequence (carrying suitable protecting groups) is attached, and so on. When the desired peptide has been completely built up, it is cleaved from the resin support, all protecting groups are removed, and the peptide is recovered. Examples of suitable protecting groups include α-tert-butyloxycarbonyl for the α-amino-group; benzyl, 4-methoxybenzyl, or 4-methylbenzyl for the thiol group of cysteine, the β-carboxylic acid group of aspartic acid, the γ-carboxylic acid group of glutamic acid and the hydroxyl groups of serine, threonine, and tyrosine; benzyloxycarbonyl or a 2-chloro- or 3,4-dimethoxy- derivative thereof for the ring nitrogens of histidine and tryptophan and the ε-amino group of lysine; p-nitrophenyl for the amide nitrogens of asparagine and glutamine; and nitro or tosyl for the guanadine group of arginine.

With regard to the cloning aspects of the invention, it is anticipated that GPIbα, or fragments thereof, could be cloned by any of the following strategies. If the cDNA sequence is available, then oligonucleotides can be chosen for PCR amplification of messenger RNA. This presumes the availability of a cell line expressing adequate levels of the

mRNA. If the mRNA is thought to be rare, subtraction hybridization schemes can be employed to amplify the desired message before performing the specific PCR amplification. The oligos could also be used to amplify the sequence desired from genomic DNA assuming that the possible existance of intron sequences can either be easily determined or will not affect subsequent use of the clone.

If antibodies directed against the protein are available, then polysomes containing the mRNA can be precipitated, the mRNA purified and copied into double stranded cDNA which can then be cloned. If a cell line abundantly expresses the protein, then first a cDNA library could be constructed using an expression vector and then the library screened by antibody binding to expressing clones.

If the protein sequence is available, then oligonucleotides can be chosen that can be used to screen cDNA or genomic libraries. A mixed set of oligonucleotides will need to be chosen since the codon usage of amino acids will not precisely be known.

Elements necessary for the practice of the preferred embodiments of the invention are: (A) DNA sequences which encode the residue His^{1}-Leu^{610} or His^{1}-Ala^{302} domains of the GPIbα polypeptide; (B) an expression plasmid or viral expression vector capable of directing in a eucaryotic cell the expression therein of the aforementioned domains; and (C) a eucaryotic host cell in which said expression may be effected.

The GPIbα polypeptides so expressed are expected not to be secreted from host cells because of the lack of attachment to the nascent GPIbα polypeptide of a signal peptide. Purification of proteins expressed therein and the extraction of pharmacologically useful quantities thereof is expected to

be more difficult than if the polypeptide could be caused to be secreted into the culture medium of the host cells. It is expected that such expression systems are nonetheless useful for diagnostic assay purposes such as testing the proper function of vWF in a patient.

Accordingly, in the preferred practice of the invention there is provided a GPIbα encoding DNA sequence for insertion into a suitable host cell in which there is inserted upstream from the residue 1-610 or 1-302 encoding sequence thereof a DNA sequence encoding the GPIbα signal peptide. Signal peptides corresponding to other protein species may prove equally effective to cause the secretion of GPIbα. von Heijne, G., J. Mol. Biol., 184, 99-105 (1985).

When attached to the amino terminal end of the residue 1-610 or 1-302 GPIb(α) polypeptide, the signal peptide causes the polypeptide to be recognized by cellular structures as a polypeptide of the kind to be processed for ultimate secretion from the cell, with concomitant cleavage of the signal polypeptide from the mature GPIbα polypeptide.

A wide variety of expression plasmids or viral expression vectors are suitable for the expression of the GPIbα polypeptides or the amino terminal regions thereof. One factor of importance in the selection of an expression system is the provision of a high efficiency transcription promoter directly adjacent to the cloned GPIbα insert.

Another factor of importance in the selection of an expression plasmid or viral expression vector is the provision of an antibiotic resistance gene marker therein so that continuous selection for stable transformant eucaryotic host cells can be applied.

Plasmids suitable in the practice of the invention include pCDM8, pCDM8^{neo}, pcDNA1, pcDNA1^{neo}, pMAM^{neo} and Rc/CMV. Plasmids whose use in the practice of the invention is preferred include pCDM8^{neo}, pcDNA1^{neo}, pMAM^{neo} and Rc/CMV. A DNA sequence encoding the GPIbα polypeptide, or a fragment thereof, may also be inserted into a plasmid or vector suitable for causing expression of the polypeptide in a bacterial system.

There are several viral expression vector systems suitable for the practice of the invention including those based upon retroviruses and those based upon baculovirus Autographa californica nuclear polyhedrosis virus.

Representative host cells comprising permanent cell lines suitable for the practice of the invention include CHO-K1 Chinese hamster ovary cells, ATCC-CCL·61; COS-1 cells, SV-40 transformed African Green monkey kidney, ATCC-CRL 1650; ATT 20 murine pituitary cells; RIN-5F rat pancreatic β cells; cultured insect cells, Spodoptera frugiperda; or yeast (Sarcomyces). Examples 9 and 10 contain a detailed explanation of preferred procedures used to express the GPIbα polypeptide or the amino terminal domain thereof.

Therapeutic compositions

One or more of the polypeptides of the present invention can be formulated into pharmaceutical preparations for therapeutic, diagnostic, or other uses. For example, to prepare them for intravenous administration, the compositions are dissolved in water containing physiologically compatible substances such as sodium chloride (e.g. at 0.05-2.0 M), glycine, and the like and having a buffered pH compatible with physiological conditions, which water and physiologically compatible substances comprise a pharmaceutically acceptable carrier.

With respect to the 45 kDa polypeptide of the invention, the amount to administer for the prevention or inhibition of thrombosis will depend on the severity with which the patient is subject to thrombosis, but can be determined readily for any particular patient.

Antibodies

Antibodies, and particularly conformation dependent antibodies, are powerful tools for analyzing the structure and function of macromolecules. By blocking macromolecular interaction, antibodies can also have important therapeutic utility. Accordingly, this invention includes within its scope an antibody which is specific for the GPIb(α) polypeptide, or any polypeptide containing one or more sequential subsets thereof, said antibody being made by a process which involves immunizing animals with a polypeptide of the invention, and isolating the antibodies generated thereby. Numerous protocols are known in the art which are suitable for immunizing animals with the polypeptides of this invention.

EXAMPLES

The following examples illustrate the biological activity of the peptides and polypeptides of the present invention and exemplary cloning methods useful in practicing the invention.

Example 1

Inhibition of Ristocetin-Induced Binding of vWF to Platelets

To test the inhibitory activity of the peptides of the present invention, formalin-fixed platelets were used at a final concentration of $1 \times 10^{11}/\ell$. Assayed peptides were then added at various concentrations. One third final volume of vWF-deficient plasma was then added followed by ^{125}I-vWF at a final concentration of 5 μg/mℓ. Ristocetin was then added at a concentration of 1.0 mg/mℓ. After incubation for 30 minutes

at room temperature, bound and free vWF ligand were separated by centrifuging 50 $\mu\ell$ of the mixture through 300 $\mu\ell$ of 20% sucrose at 12,000 g for 4 minutes. The platelet pellet was then separated from the rest of the mixture to determine platelet-bound radioactivity. Nonspecific binding was defined as the residual binding of ^{125}I-vWF in the presence of a 50-fold excess of unlabeled vWF but in the absence of any peptides.

Percent inhibition with a given peptide was calculated by dividing the specific cpm in the absence of peptide. The IC_{50} values (concentration of peptide which inhibited binding by 50%) for the peptides tested are shown in Table IV, below.

Table IV

$(KR)_7$	9 and 15 μM (two experiments)
$(KR)_5$	13 μM
$(KR)_3$	120 μM
$(KR)_2$	200 μM
$(KR)_4$GDV	16 μM
$(R)_8$GDV	6 μM
YRGDV	> 600 μM (no inhibition seen)

Complete inhibition was not seen with any of the peptides at the concentrations tested.

Example 2

Inhibition of Asialo-vWF Binding to Fresh Platelets

Fresh platelets were prepared by drawing blood into a solution of 11 mM trisodium citrate and 2 mM EDTA. Platelet-rich plasma was then prepared by differential centrifugation. The platelet count was then adjusted to 1 x $10^{11}/\ell$. Peptides were then added to various concentrations and ^{125}I-asialo-vWF was then added at a final concentration of 5 μg/mℓ. After incubation for 30 minutes at room temperature, bound and free vWF ligand were separated by centrifuging 50 $\mu\ell$ of the mixture through 300 $\mu\ell$ of 20% sucrose at 12,000 g for 4 minutes. The platelet pellet was then separated from the rest of the

mixture to determine platelet-bound radioactivity. Non-specific binding was defined as the residual binding of ^{125}I-asialo-vWF in the presence of a 50-fold excess of unlabeled vWF but in the absence of any peptides.

Percent inhibition with a given peptide was calculated by dividing the specific cpm obtained when various concentrations of peptide were added by the specific cpm in the absence of peptide. The IC_{50} values (concentration of peptide which inhibits binding by 50%) for the peptides tested are shown in Table V, below.

Table V

$(KR)_7$	1.5 μM
$(KR)_5$	1.7 μM
$(KR)_3$	23 μM
$(KR)_4$GDV	15 μM
$(R)_8$GDV	3.5 μM
$(R)_{11}$	7 μM

Complete inhibition was seen at the following concentrations, shown in Table VI, below.

Table VI

$(KR)_7$	12 and 15 μM
$(KR)_5$	6 and 7 μM
$(KR)_3$	60 and 120 μM
$(KR)_4$GDV	44 μM
$(R)_8$GDV	24 μM

Example 3

Inhibition of Ristocetin-Induced Platelet Aggregation

The inhibitory activity of the peptides of the present invention was evaluated using washed platelets. The platelets, prepared as described in Trapani-Lombardo et al., J. Clin. Invest., **76**, 1950-1958 (1985), were adjusted to a final concentration of 3×10^{11}. Peptides, at varying concentrations, and purified vWF, at a final concentration of

0.8 μg/mℓ, were incubated with the platelets for 5 minutes at 37°C. Ristocetin was then added at a final concentration of 1.0 mg/mℓ. Reaction mixtures were prepared in siliconized glass cuvettes and then placed in a Lumi aggregometer (Chrono-Log Corp.) at 37°C with constant stirring of the platelet suspension at 1200 rpm. Aggregation was quantitated by monitoring the increase in light transmittance through the stirred platelet suspension.

The IC_{50} values (the concentration which inhibited aggregation by 50%, as judged by the percent decrease in the initial slope of the aggregation curve) of the peptides are shown in Table VII, below.

Table VII

$(KR)_7$	3 μM
$(KR)_5$	50 μM
$(KR)_3$	250 μM

At a concentration of 100 μM the following peptides inhibited aggregation to the extent shown in Table VIII, below.

Table VIII

$(KR)_7$	82% inhibition
$(KR)_5$	50% inhibition
$(KR)_3$	no significant inhibition
$(R)_8GDV$	70% inhibition

Example 4

Inhibition of Asialo-vWF-Induced Aggregation

The inhibitory activity of the peptides of the present invention was determined using platelet-rich plasma prepared by differential centrifugation of blood drawn into 11 mM trisodium citrate anticoagulant. The platelet count was adjusted to $3 \times 10^{11}/\ell$. The peptides, at a concentration of 55 μM, were incubated with platelet rich plasma for 5 minutes at

37°C. Asialo-vWF was then added at a final concentration of 15 μg/mℓ. Reaction mixtures were prepared in siliconized glass cuvettes and then placed in a Lumi aggregometer (Chrono-Log Corp.) at 37°C with constant stirring of the platelet suspension at 1200 rpm. Aggregation was quantitated by monitoring increase in light transmittance through the stirred platelet suspension.

Inhibition of aggregation by the peptides is shown in Table IX, below.

Table IX

$(KR)_7$	100% inhibition
$(KR)_5$	88% inhibition
$(KR)_3$	no significant inhibition
$(R)_8GDV$	92% inhibition

Example 5

Identification of GPIbα Receptor Sites

It has been previously demonstrated that the amino terminal extracytoplasmic region of the GPIbα chain, extending between residues 1 and 293, contains a domain or domains which interact with vWF in the absence of any other component of the GBIb complex, or any other platelet membrane component, Vicente et al.

The studies of the present application were designed to identify the receptor sites of this interaction. The entire amino acid sequence of this 45 kDa binding fragment was reproduced as a series of 27 overlapping synthetic peptides which were used in vWF binding inhibition assays.

Percent inhibition of binding of vWF to platelets was measured using I^{125} labelled vWF prepared according to the methods of Ruggeri et al. See also DeMarco et al., J. Clin. Invest., **68**, 321-328 (1981). Binding of vWF to platelets

induced by ristocetin and/or botrocetin (and inhibition thereof) was measured according to the method of MacFarlane et al., Thromb. Diath. Haemorrh., **34**, 306-308 (1975) which utilizes washed platelets fixed with formaldehyde. The top part of Figure 1 shows the amino acid sequence of the amino terminal region of GPIbα in one-letter notation. T_1 indicates the site of tryptic cleavage that gives origin to the 45 kDa domain. Numerals above the sequence line indicate the first residue in a synthetic peptide sequence, and the same number below the sequence line indicates the last residue in that peptide. The heavy bar underlines the sequence of the longer peptide (29 residues) used in subsequent studies. The lower part of the figure displays in a bar graph the inhibitory effect of all the peptides tested on ristocetin-dependent (black bars) and botrocetin-dependent (hatched bars) vWF binding to GPIb-IX. Each peptide, used at a final concentration of 500 μmol/ℓ with a ^{125}I-vWF concentration of 2 μg/mℓ, is identified by the same numeral used in the top part of the figure. Note that ristocetin-dependent binding was inhibited by five groups of peptides (mainly those identified by numbers 3-4, 7-9, 14, 21, and 23-25), while botrocetin-dependent binding was significantly inhibited only by peptides 7-10 and 19-25. Peptide 25 shows the most promise as an inhibitor based on both assay systems.

Example 6

Activity Assay of the GLY^{271} to GLU^{285} Fragment

Reference to Figure 1, discussed in the previous example, indicates that peptide 25, representing the sequence GLY^{271} to GLU^{285} of glycocalicin, shows excellent promise as an inhibitor of vWF binding.

Further experiments were performed with a constant ^{125}I-vWF concentration of 2μg/mℓ and varying peptide concentration, as

indicated on the Figure 2 abscissa. The top panel shows the mean and range of three determinations of residual vWF binding in the presence of botrocetin; the lower panel, mean and range of five determinations in the presence of ristocetin. Residual binding was calculated after subtracting from each experimental point the value measured in the presence of a saturating amount of the anti-GPIb monoclonal antibody LJ-Ibl. One hundred percent binding was that measured in the presence of Hepes buffer instead of peptide.

Example 7

Activity Assay of the SER^{251} to TYR^{279} Fragment

Reference to Figure 1 (corresponding to example 5) demonstrates that peptides 23 through 25 have improved binding relative to synthetic peptide 26. Since the amino acid sequence of peptides 26 and 25 overlap, it was postulated that constructing a peptide which omitted the terminal portion of peptide 26 would yield a peptide showing significant inhibitory activity. Accordingly a synthetic peptide constituting the glycocalicin sequence SER^{251} through TYR^{279} was constructed and tested.

Binding inhibition experiments were conducted analogous to those in Example 6 using an I^{125}-vWF concentration of 2 $\mu g/m\ell$. The mean and range of two separate binding inhibition experiments are indicated in Figure 3.

Comparison of the various peptides mentioned in the above examples shows that the most active of the original 26 synthetic peptides is number 25 which inhibits 50% of ristocetin-induced binding of vWF at a concentration of 420 μM, and 50% of botrocetin-induced binding at 530 μM. In contrast, the peptide of this example, representing residues 251-279, inhibits 50% of ristocetin induced binding at a concentration of 170 μM.

The basis for designing synthetic polypeptide inhibitor molecules which have a high probability to bind vWF (because the selected domains occur on the surface of the native GPIbα chain), and which use inert linker sequences to connect specific glycocalicin domains, is demonstrated by the method of Emini et al., J. of Virology, **55**, 836-839 (1985). These surface probability index calculations show that 13 of the 15 amino acids in peptide 25, and 12 positions in the peptide of this example, have a surface probability index greater than four.

It is further postulated that since the peptide of this example contains a cysteine capable of dimerization, and dimers are indeed the prevalent form under the temperature, pH, and time conditions of vWF binding assays, as shown by reverse phase HPLC, that such dimerization confers upon the peptide structural alterations which enhance its binding to vWF when compared to non-dimerized peptides.

Example 8

Design of Complex Synthetic Polymers

Reference to Figure 1 and Example 5 demonstrates that peptide 8 and peptide 25 represent effective domains from which to design complex synthetic polymers containing vWF binding regions and inert spacer or linker sequences so that multimeric vWF complexes can be maximally inhibited.

Example 9

GPIbα (His^{1}-Leu^{610}) Expression in Stable Mammalian Transformants

Step 1. Construction of a DNA sequence for expression of the mature His^{1}-Leu^{610} polypeptide

Based on the published GPIbα cDNA sequence of Lopez et al., two flanking oligonucleotides were synthesized for the amplification in a polymerase chain reaction of a region of

the GPIbα gene which it was believed would be suitable as a probe to screen a human genomic lambda (λ) phage library.

Accordingly, human genomic DNA was subjected to enzymatic amplification in a polymerase chain reaction according to the method of Saiki et al., Science, **239**, 487-491 (1988). The procedure utilizes a double stranded GPIbα DNA sequence, a subsegment of which is to be amplified, and two single stranded oligonucleotide primers which flank the ends of the subsegment. The primer oligonucleotides (in the presence of a DNA polymerase and deoxyribonucleotide triphosphates) were added in much higher concentrations than the DNA to be amplified. The vast majority of polynucleotides which accumulate after numerous rounds of denaturation, oligonucleotide annealing, and synthesis represent the desired double stranded cDNA subsegment suitable for further propagation by cloning.

PCR reactions were performed with a DNA thermal cycler (Perkin Elmer Co., Norwalk, CT/Cetus Corporation, Berkeley, CA) using Taq polymerase (Thermus aquaticus). The reactions were run in 100 μℓ volumes containing 1.0 μg of human genomic DNA, 1.0 μg of each synthetic oligonucleotide primer, and buffer consisting of 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1% gelatin (BioRad Co., Richmond, CA) and 200 mM of each dNTP. PCR conditions were 35 cycles of 30 seconds at 94°C, 30 seconds at 52°C and 1 minute at 72°C. Amplified fragments were then purified and isolated by electrophoresis through a 2% agarose gel, Maniatis et al., Molecular Cloning, A Laboratory Manual, 164-170, Cold Spring Harbor Lab., Cold Spring Harbor, NY (1982).

Specifically, the following oligonucleotides were synthesized by the phosphoramidite method, Sinha et al.,

Tetrahedron Letters, **24**, 5843 (1983) using a model 380B automated system, Applied Biosystems, Foster City, CA.

The oligonucleotides selected were:

Oligonucleotide (A)

```
          644                                                  674
           ↓                                                    ↓
5' - G AAT CTG ACA GCG CTG CCT CCA GAC CTG CCG - 3'
     Asn                                                    Pro
      21                                                     30
```

and

Oligonucleotide (B)

```
3' - GC GAC GGA GAA CCA CGG GAC - 5'
     ↑                         ↑
    925                       944

5' - cg ctg cct ctt ggt gcc ctg - 3'
        Leu                 Leu
        115                 120
```

Oligonucleotide (A) is equivalent to non-transcribed strand DNA (coding strand) for nucleotides 644-674 (using the numbering system of Wenger et al. for the GPIbα gene).

Oligonucleotide (B) is shown 3' → 5' and is equivalent to the transcribed strand (noncoding DNA). The corresponding coding strand is shown 5' → 3' in lower case letters. Nucleotide positions are according to Wenger et al.

T_4 kinase was used to add phosphate groups to each end of the amplified fragment. T_4 ligase was used to blunt end ligate the fragment into the SmaI site within the multiple cloning sequence of the double stranded replicative form of M13mp18 bacteriophage. The ability to isolate a stable single stranded (+) form of the virus is particularly useful to verify the integrity of any cloned sequences therein. See

Messing, J. Meth. Enzymology, **101**, 20-78 (1983), and Yanish-Perron et al., Gene, **33**, 103-109 (1985). Accordingly, the GPIbα DNA insert was completely sequenced using single stranded dideoxy methodology (Sanger et al., Proc. Natl. Acad. Sci. USA, **74**, 5463-5467 (1977) utilizing the single stranded (+) form of M13mp18. Sequencing in M13mp18 established that the GPIbα insert was 301 base pairs in length, indicating that the corresponding cDNA region, Lopez et al., did not involve an intron boundary. The 301 base pair (bp) fragment was then subjected to nick translation for incorporation of ^{32}P-labelled nucleotides, thus converting the fragment into a radiolabelled probe, Rigby et al., J. Mol. Biol., **113**, 237 (1977).

A human genomic λ phage library (using Lambda Fix™, Stratagene, La Jolla, CA) was prepared using an EcoRI partial digest of human cell DNA. The library was screened following the hybridization and plaque purification procedure of Benton et al., Science, **196**, 180-182 (1977) using E. coli strain LE 392 as host. Screening with the 301 bp fragment resulted in the isolation of 6 positive clones after 4 cycles of plaque purification.

In order to conduct the library screening for each positive clone, an appropriate dilution of λ phage was incubated with bacteria at 37°C for 20 minutes with constant shaking. Melted agarose was added to this mixture and the entire contents spread onto a petri dish with a hard agar base. The plates were incubated overnight at 37°C. An imprint of the bacteriophage plaques thus obtained was produced by gently placing a nitrocellulose filter onto the surface of the plate. Phage particles and DNA were transferred to the filter by capillary action in an exact replica of the pattern of plaques. After denaturation with NaOH, the DNA was irreversibly bound to the filter by baking and was then hybridized to the ^{32}P-labelled probe. Unbound probe was washed

away and the filters were exposed to film. Plaques which were positive for hybridization were identified by aligning the film with the original agar plate. These plaques were individually picked and amplified. In general the initial plating density of phage was such that individual plaques could not be picked but instead an area comprising several different phage species was picked. This mixture was amplified and replated at low density to be rescreened to determine which initial positives were true positives and to "plaque" purify each positive. After 3 rounds of such rescreening individual positively hybridizing phage were isolated for further characterization.

Purified λ DNA was then isolated from each positive λ clone by precipitating phage from respective lysed E. coli LE 392 samples following the procedure of Maniatis et al., at 76-85.

One μg samples of DNA from each of the six positive λ clones were then digested with EcoRI. The EcoRI digests were then separated according to molecular weight by electrophoresis in agarose, followed by transfer to nitrocellulose for detection by autoradiography using the ^{32}P-labelled 301 bp fragment. Southern, J. Mol. Biol., **98**, 503 (1975). An approximate 6000 base pair EcoRI fragment was recognized.

The approximate 6000 base pair fragment visualized and extracted from an agarose gel was then cloned into pBluescript KS- plasmid (Stratagene Co., La Jolla, CA) at its EcoRI site. The plasmid was then propagated in E. coli strain XL-1 Blue (Stratagene Co.). Plasmids were recovered from host E. coli by an alkaline cell lysis procedure, Birnboim and Doly, Nucleic Acids Research, **7**, 1513 (1979) followed by purification by CsCl/ethidium bromide equilibrium centrifugation according to Maniatis et al., at 1.42.

Plasmid so isolated was then digested with BamHI and BglII creating a 2161 base pair fragment (nucleotides 503 to 2663 using the numbering system of Wenger et al.) which fragment extends from upstream above the initiating MET^{1} codon (nucleotides 537-539) to downstream below the LEU^{610} codon (nucleotides 2412-2414) and the TGA translation stop codon (2415-2417). The BamHI site of the fragment corresponds to nucleotides 502-507 and the BglII site thereof nucleotides 2658-2664.

The 2161 bp fragment was then cloned into the BamHI site of pBluescript KS- (Strategene Co., La Jolla, CA) as a BamHI-BglII fragment. Since BamHI and BglII restriction sites contain identical internal sequences GATC/CTAG, a BglII restricted site may be annealed into a BamHI site. The fragments were ligated with T_4 DNA ligase, however the integrity of the affected BglII end was not restored. Hybridization with the 301 base pair probe and sizing on agarose were repeated. The plasmids were propagated in E. coli XL-1 Blue.

Restriction mapping was then performed to select a clone of E. coli XL-1 Blue (Stratagene) in which the GPIbα DNA within a contained pBluescript KS- plasmid possessed an insert orientation such that the XhoI site of the polylinker would be upstream (5') from the insert and the NotI site would be downstream (3') therefrom. The XhoI-NotI fragment was used as follows to create a suitable expression plasmid.

Step 2. Construction of plasmids for integration into mammalian cells

A selection procedure based on aminoglycosidic antibiotic resistance was designed to select, continuously, for transformants which would retain a suitable GPIbα expression plasmid.

pCDM8 vector, (developed by Seed et al., Nature, **329**, 840-842 (1987) and available from Invitrogen, San Diego, CA) was modified by Dr. Timothy O'Toole, Scripps Clinic and Research Foundation, La Jolla, CA to include a neomycin resistance gene (phosphotransferase II) that was cloned into the BamHI restriction site of pCDM8 as a part of a 2000 base pair BamHI fragment. The protein produced by the neomycin (neo) gene also confers resistance against other aminoglycoside antibiotics such as Geneticin® G418 sulfate (Gibco/Life Technologies, Inc., Gaithersburg, MD).

Several other suitable expression vectors containing neomycin resistance markers are commercially available. Examples include pcDNA 1^{neo} (Invitrogen, San Diego, CA), Rc/CMV (Invitrogen, San Diego, CA) and $pMAM^{neo}$ (Clontech, Palo Alto, CA). If necessary the GPIbα fragment may be differently restricted or modified for expression capability in these other expression plasmids.

The XhoI-NotI fragment from pBluescript KS- plasmid was inserted into $pCDM8^{neo}$ which had been restricted with XhoI and NotI. Ampicillin sensitive E. coli strain XS-127 cells (Invitrogen, La Jolla, CA) were transformed with the resultant ligated DNA mixture following the method of Hanahan, J. Mol. Biol., **166**, 557-580 (1983).

Plasmids from resultant colonies were characterized by restriction mapping and DNA sequencing to identify colonies which contained the intended insert. One such plasmid (designated pMW1), was maintained in E. coli strain XS-127, and was selected for mammalian cell transformation procedures.

Prior to use in transforming mammalian cells, supercoiled plasmids (pMW1) were recovered from host E. coli by the alkaline cell lysis procedure of Birnboim and Doly followed by

purification by CsCl/ethidium bromide equilibrium centrifugation according to Maniatis et al., at 1.42.

Step 3. Transformation of Chinese hamster ovary cells

pMW1 was introduced into CHO-K1 Chinese hamster ovary cells (ATCC-CCL·61) by a standard calcium phosphate-mediated transfection procedure. Chen et al., Mol. Cell. Biol., 7(8), 2745-2752 (1987).

CHO-K1 cells were grown to confluence at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Gibco/Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), 0.5 mM of each nonessential amino acid (from NEAA supplement, Whittaker, Walkersville, MD) and 2.5 mM L-glutamine under a 5% CO_2 atmosphere, trypsinized as elaborated below, and then subcultured 24 hours prior to transformation at a density of 1.25×10^5 cells per 60 mm tissue culture dish (approximately 25% of confluence). CHO-K1 cells have a doubling time in DMEM/10% FCS of approximately 16 hours under these conditions.

To accomplish transformation, pMW1 plasmids were recovered from cultures of E. coli strain XS-127, according to the method of Birnboim and Doly. 10 μg of plasmids was applied to the cells of each 60 mm dish in a calcium phosphate solution according to the method of Chen et al. After inoculation with plasmid the cells were maintained in DMEM/10% FCS at 37°C in a 5% CO_2 atmosphere.

Approximately 48 hours post-transfection and after growth at 37°C in a 5% CO_2 atmosphere, the cells were trypsinized as follows. Growth medium for each dish was replaced by 3 mℓ of a solution of phosphate-buffered saline (37 mM NaCl, 27 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$/1.4 mM K_2HPO_4, pH 7.4) containing also 0.25% trypsin, 0.2% (w/v) EDTA. Trypsinization was conducted

for 3 minutes. The trypsin-containing medium was removed and the dishes were then placed in the incubator for a further 15 minutes after which the cells were resuspended in DMEM containing 10% FCS. The cells from each dish were then split 20 fold, and plated at a density of approximately 1.2 x 10^4 cells/60 mm dish (approximately 2% of confluence).

Production of stable transformants, which have integrated the plasmid DNA, was then accomplished by adding Geneticin® G418 sulfate to the 60 mm dishes to a concentration of 0.8 mg/mℓ. Growth was continued for 14 days at 37°C in a 5% CO_2 atmosphere. Surviving independent colonies were transferred to 12 well plates using cloning rings and then grown for another seven days in DMEM/10% FCS supplemented with 0.8 mg/mℓ of Geneticin®. Under these conditions 3 to 7 surviving colonies per plate were apparent after 10-14 days. Approximately 100 stable transformants can be isolated from each original 60 mM dish originally containing approximately 5 x 10^5 cells at a plate density of approximately 70% of confluence.

Based on screening with the LJ-P3 anti-GPIbα monoclonal antibody, more than 50 percent of G418-resistant cell lines produce antigen corresponding to mature GPIbα polypeptide. The specific geometry of integration of each clone presumably prevents expression in all cases. Stable transformants were then cultured and maintained at all times in medium containing Geneticin® G418 sulfate (.8 mg/mℓ) to apply continuous selection.

Colonies expressing the recombinant mature GPIbα polypeptide were detected by dot-blot analysis on nitrocellulose after lysis in buffer. As a control, recombinant cell extracts were compared with that from nontransfected CHO-K1 cells.

To prepare cell extracts, non-transfected or transfected CHO-K1 cells were harvested with 3.5 mM EDTA and resuspended in 0.25 M Tris-HCl pH 7.5 (10^3 cells/$\mu\ell$). Cells were lysed by three cycles of freezing and thawing and centrifuged at 12,000 g to remove cell debris. The resulting supernatant was kept at -70°C as cell extract.

To prepare samples of culture medium containing secreted GPIbα antigen, 80% confluent non-transformed or transformed CHO cells grown in medium containing FCS were washed once with serum-free medium, and then fed with serum-free medium supplemented with L-glutamine and nonessential amino acids. After 24 hours, the medium was collected and centrifuged at 12,000 g to remove cell debris. The corresponding supernatants were pooled and stored at -70°C until used. Monoclonal antibodies LJ-Ib1 Handa et al. and LJ-P19, which recognize GPIbα native conformation were used as primary antibody. The secondary antibody (^{125}I-rabbit anti-mouse IgG) which had been labelled by the method of Fraker et al., Biochem. Biophys. Res. Commun., **80**, 849-857 (1978) was incubated for 2 hours at 25°C on a nitrocellulose sheet. After rinsing, the nitrocellulose was developed by autoradiography to identify colonies expressing GPIbα antigen.

Extracts from pMW1-transformed cells contain as a minor component a glycoprotein Ibα antigen having an approximate apparent molecular weight of 79 kDa as measured by SDS-polyacrylamide gel electrophoresis under reducing or non-reducing conditions. This band represents full length glycoprotein Ibα chain (residues 1-610 and minus the signal peptide) without glycosylation. The 79 kDa polypeptide reacts with anti-GPIbα monoclonal antibody LJ-IBα1 which has its epitope in the amino terminal region of the denatured (α) polypeptide, whether in reduced or unreduced form. The relatively small proportion of this species indicates its

inherent instability and rapid proteolytic processing. Components of oligomeric membrane complexes (such as GPIbα·GPIbβ·GPIbIX) that fail to assemble properly are not transported beyond the endoplasmic reticulum and are degraded intracellularly. Thus expression of the (α) gene without simultaneous expression of the (β) and (IX) genes is not expected to result in the isolation of an (α) polypeptide or biologically active forms thereof. (Lopez, J.A. et al., Circulation, 82(4), 597a (1990), Krangel, M.S. et al., Cell, 18, 979-991 (1979), Woods, C.M. et al., Cell, 40, 959-969 (1985), Minami, Y. et al., Proc. Natl. Acad. Sci. USA, 84, 2688-2692 (1987)).

As expected, the 79 kDa polypeptide was not detected in culture medium from pMW1 transformed cells. Instead the major GPIb(α) polypeptide isolated from such medium has an approximate apparent molecular weight of 45 kDa, characteristic of the properly glycosylated amino terminal domain of GPIbα. The presence of this species in the culture medium of pMW1 transformed cells demonstrates that the amino terminal domain of GPIbα can be processed as a secretory protein and reaches structural maturation (A) in the absence of assembly of the other components of the GPIb complex and (B) in spite of the usual proteolysis of the full length GPIbα polypeptide.

It is anticipated, however, that stable cell lines will be found which allow for more substantial expression of the full length polypeptide, and the proper folding and glycosylation thereof. As demonstrated below, the His^{1}-Ala^{302} fragment contains sufficient primary sequence information to be assembled into a structure possessing domains of tertiary structure present in native glycoprotein Ibα. It is expected that the expression of a polypeptide containing the amino acid sequence from approximately His^{1} to approximately Ala^{302}, and

additional GPIbα sequence on the carboxy terminal side of Ala^{302}, will also result in a polypeptide possessing the biological activity of the 45 kDa fragment.

Example 10

Expression of a His^{1}-Ala^{302} GPIbα Fragment in Stable Mammalian Transformants

This example demonstrates conditions under which a DNA sequence encoding the fragment of mature GPIbα polypeptide having an amino terminus at His^{1} and a carboxy terminus at residue Ala^{302} thereof may be expressed in and secreted from cultured mammalian cells.

The following section concerns primer directed amplification of DNA. pBluescript KS- containing at its BamHI site the 2161 base pair fragment (nucleotides 503-2664 according to Wenger et al.) was subjected to enzymatic amplification in a polymerase chain reaction according to the method of Saiki et al., and following generally the procedures of Example 9, above.

The following oligonucleotides were synthesized by the phosphoramidite method, Sinha et al., using a model 380B automated system, Applied Biosystems, Foster City, CA.

Nucleotides are shown using the numbering system of Wenger et al. for the GPIbα gene.

Oligonucleotide (C)

```
          502                    525
           ↓                      ↓
5'  GGATCCACTCAAGGCTCCCTTGCC  3'
     BamHI
```

Oligonucleotide (D)

3' CAG TTC AAG GGG TGG TTT CG 5' ← link with BamHI

```
5'   gtc aag ttc ccc acc aaa gc   3'
     Val                   Ala
     296                   302
```

(nucleotide positions 1470-1489)

Oligonucleotide (C) is equivalent to nontranscribed (coding) strand DNA. Oligonucleotide (D), shown in capital letters, is equivalent to transcribed (noncoding) strand DNA. The corresponding coding strand for oligonucleotide (D) is shown 5' → 3' with the encoded amino acids shown by standard three letter designation.

A BamHI linker was added to the amplified double stranded DNA sequence 3' to the partial Ala^{302} codon thereby completing the codon and enabling the DNA to function as a BamHI insert. Roberts et al., Nature, **265**, 82-84 (1977).

The amplified fragment was then cloned into the BamHI site within the multiple cloning sequence of the double stranded replicative form of M13mp19 bacteriophage. The ability to isolate a stable single stranded (+) form of the virus is particularly useful to verify the integrity of any cloned sequences therein. See, for example, Messing and Yanish-Perron et al.

Accordingly, the GPIbα DNA insert was completely sequenced using single stranded dideoxy methodology, Sanger et al., utilizing the single stranded (+) form of M13mp19 to confirm that the GPIbα fragment contained the correct coding sequence for the region of GPIbα DNA represented by nucleotides 502 to 1489 and including a codon for the initiating methionine, the remaining 15 residues of the signal peptide and residues 1 to 302 of the amino terminal region of mature GPIbα polypeptide.

Sequencing in M13mp19 established numerous clones having insert orientation at the BamHI site suitable for expression from pCDM8^{neo} plasmid. The GPIbα sequence of one such clone was removed from M13mp19 as an EcoRI (5') - XbaI (3') fragment which was then cloned into the polylinker region of pBluescript KS-. An XhoI (5') - NotI (3') fragment of this second insert was then removed from pBluescript KS- and cloned into pCDM8^{neo}, which had been restricted with Xho and NotI, following the procedures used for insertion of pMW1 (see Example 9).

Ampicillin sensitive E. coli strain SX-127 cells (Invitrogen, San Diego, CA) were transformed with the resultant ligated DNA mixture following the method of Hanahan.

Plasmids from resultant colonies were characterized by restriction mapping and DNA sequencing to identify colonies which contained the intended insert. One such appropriate plasmid (designated pMW2) was maintained in E. coli strain XS-127, and was selected for mammalian cell transformation procedures.

Prior to use in transforming mammalian cells, supercoiled plasmids (pMW2) were recovered from host E. coli by the alkaline lysis procedure of Birnboim and Doly followed by CsCl/ethidium bromide equilibrium centrifugation according to the procedure of Example 9. Transformation of CHO-K1 cells also followed the procedure of Example 9 for pMW1 plasmid.

Example 11

Demonstration of Native Tertiary Structure in the Polypeptide Produced by pMW1 and pMW2 Plasmids

The presence of GPIbα antigen in stable transformant cells (containing pMW1 or pMW2 plasmid) was demonstrated by applying

cell lysates or culture medium from CHO-K1 containing dishes (both prepared as in Example 9) to nitrocellulose.

10 μℓ aliquots of lysate or culture medium were spotted onto nitrocellulose membranes (.45 micron pore size, Bio-Rad, Richmond, CA) and air dried. The membrane was then soaked with constant shaking for 2 hours at 22-25°C in "Blotto" (5 mg/mℓ fat-free dry milk, 0.25 mM phenylmethyl sulfonyl fluoride, 0.15 M NaCl in phosphate buffer pH 7.3), a protein blocking solution to inhibit nonspecific interaction.

The membrane was then incubated with native GPIbα conformation-requiring monoclonal antibody (5-10 μg/mℓ of LJ-Ib1 or LJ-P19) for two hours at 22-25°C. After washing 3 times with Blotto, the membrane was transferred to a solution of ^{125}I-labelled rabbit anti-mouse IgG (0.08-0.16 mCi I^{125} per dot) and incubated for 2 hours at 22-25°C. The wash with Blotto was repeated 3 times prior to drying and making the autoradiograph (using Kodak AR film).

Figure 4 demonstrates results with LJ-Ib1 and LJ-P19 primary antibody using cell extract or culture medium from pMW1 and pMW2 transformed cells. Cell extract and culture medium from untransformed CHO cells were used as controls. Figure 4 demonstrates that rIbα1 antigen and rIbα2 antigen (produced by pMW1 and pMW2 transformants respectively), whether isolated from cell lysates or culture medium, present domains of tertiary conformation present in native GPIbα. Similar results were obtained using another conformation dependent anti-GPIbα monoclonal antibody, LJ-P3.

Example 12

Intracellular Processing of the GPIbα Polypeptide Produced by pMW2 Plasmid

Polypeptides produced by pMW2-transformed CHO-K1 cells from a representative cell line were characterized under reducing conditions by immunoblotting ("Western blotting") following the procedure of Handa et al. See also Burnett et al., A. Anal. Biochem., **112**, 195-203 (1981).

The disulfide bonds of the pMW2 polypeptides were reduced prior to electrophoresis by treatment with 30 mM dithiothreitol at 37°C for 1 hour in the absence of denaturing agents. Electrophoresis was performed on a sodium dodecylsulfate polyacrylamide 10% gel (SDS-PAGE) and protein samples were then stained with Coomassie Brilliant Blue. Protein bands from duplicate gels were transferred to nitrocellulose (.45 micron pore size, BioRad, Richmond, CA) using 350 milliamperes per gel at 3°C for 18 hours. GPIbα antigenic material was visualized by first incubating the nitrocellulose membrane with LJ-Iba1 monoclonal antibody, the epitope of which has been previously identified on the reduced 35 kDa amino terminal fragment of GPIbα, Vicente et al.

Immunoreactive bands were visualized using ^{125}I- rabbit anti-mouse IgG as secondary antibody, labelled by the method of Fraker et al.

Extracts from CHO-K1 cells transformed with pMW2 plasmid which were run under reducing conditions reveal a prominent precursor polypeptide species of approximately 60 kDa apparent molecular weight probably posessing incompletely processed precursor carbohydrate(Figure 5). Polypeptide from culture medium tested under similar conditions is revealed as a band of approximate 45 kDa apparent molecular weight, consistent with the known weight of the amino terminal 45 kDa tryptic fragment. Extracts from platelets reveal the expected full length 145 kDa GPIbα polypeptide.

Example 13

Botrocetin-Induced Binding of ^{125}I-vWF to the GPIb(α) Polypeptide Produced by pMW2 Plasmid

It has been demonstrated that botrocetin, extracted from the venom of Bothrops jararaca, modulates the in vitro binding of multimeric von Willebrand factor to platelets (Read, et al. Proc. Natl. Acad. Sci., 75, 4514-4518 (1978)) and that botrocetin binds to vWF within the region thereof containing amino acid sequence positions 441-733 (of the mature subunit), and thus the GPIb binding domain. (Andrews, R.K. et al., Biochemistry, 28, 8317-8326 (1989)). This example demonstrates that the His^{1}-Ala^{302} polypeptide produced by CHO-K1 cells stably transformed with pMW2 plasmid is functionally active.

Fifty μℓ volumes of culture media (DMEM without FCS) from pMW2 transformed CHO-K1 cells (at or near confluence) were placed in microtiter wells with circular nitrocellulose membranes (8 mm diameter) and incubated at room temperature for 30 minutes. The filters were then washed two times with a solution of 20 mM Hepes, pH 7.4, 150 mM NaCl, and 6% bovine serum albumin (HEPES/BSA). To minimize background caused by nonspecific interaction, blocking with HEPES/BSA was continued for 2 days at 4°C.

To initiate the assay, 30 μℓ volumes of monoclonal anti-GPIbα antibody (resulting in specified final concentrations thereof, Figure 6) were incubated with the culture medium-coated nitrocellulose membranes for 15 minutes at room temperature. A mixture comprised of 10 μℓ of ^{125}I-vWF and 10 μℓ of botrocetin (Sigma, St. Louis, MO) was preincubated for 5 minutes at room temperature and then added to the microtiter wells for a further 15 minute incubation. The resultant botrocetin concentration was 5 μg/mℓ. The filters were then washed 4 times with HEPES/BSA. Bound ^{125}I radioactivity was

then determined for each nitrocellulose filter to measure botrocetin-induced vWF binding to rIbα2.

Figure 6 demonstrates the functional activity of the His^{1}-Ala^{302} GPIbα polypeptide. Anti-GPIbα monoclonal antibody LJ-Ib1 (100 μg/mℓ) and LJ-Ib10 (100 μg/mℓ) substantially inhibit rIbα2 polypeptide-vWF interaction as predicted by the fact that LJ-Ib1 and LJ-Ib10 are known inhibitors of GPIbα-vWF interaction. LJ-Ib1 recognizes a native conformation-dependent epitope of GPIbα. Handa, et al., and also Vicente, et al.

Figure 6 demonstrates also that monoclonal antibodies "LJ-P3" and "229" do not inhibit rIbα2-vWF interaction. This is expected since although antibodies LJ-P3 and 229 have epitopes on GPIbα and vWF respectively, they inhibit the binding of vWF to platelets only very weakly.

Example 14

Ristocetin-Induced Binding of ^{125}I-vWF to the GPIb(α) Polypeptide Produced by pMW2 Plasmid

This example also demonstrates that the His^{1}-Ala^{302} polypeptide produced by CHO-K1 cells stably transformed with pMW2 plasmid is functionally active. To perform the assays, a device used for the enzyme-linked immunofiltration technique (ELIFA) was adapted in combination with immobilization of recombinant pMW2 polypeptide. The 45 kDa GPIbα fragment was immobilized onto a nitrocellulose membrane (0.45 μ pore size) placed at the interface between a 96-well sample application plate and a vacuum chamber. Commercially available filtration and pump materials were used.

Immobilization of the 45 kDa fragment was accomplished by causing a 200 μl volume of culture medium from pMW2-transformed CHO cells to be vacuum-drawn through the

nitrocellulose membrane over a 5 minute period. The protein binding capacity of the membrane was then saturated by passing through it three consecutive 200 μl aliquots of HEPES/BSA buffer, herein comprising 20 mM Hepes, pH 7.4, 150 mM NaCl, and 1% w/v bovine serum albumin (Calbiochem, La Jolla, CA).

After completion of the above procedure to minimize background caused by nonspecific interaction, a 50 μl volume of HEPES/BSA containing ^{125}I-vWF which had been preincubated therein with ristocetin (Sigma Chemical Co., St. Louis, MO) was vacuum drawn through the nitrocellulose membrane again over a 5 minute period. Preincubation in the 50 μl aliquot was accomplished at room temperature for 30 minutes using various concentrations of ristocetin (0-2.0 mg/ml) and a specified amount of ^{125}I-vWF (0.25 μg/ml having a specific activity of 1.13 x 10^9 cpm/mg).

The membrane was then allowed to dry and discs corresponding to the position of each application well were cut out and counted in a γ scintillation spectrometer to determine bound radioactivity. An autoradiograph of the membrane was also obtained before cutting out the discs in order to ascertain that there was no leakage of radioactivity from one well to another.

^{125}I-vWF radioactivity bound was determined as a function of the ristocetin concentration measured in the preincubation mixture. In the control (no ristocetin) only approximately 100 counts per minute (cpm) were detected per well whereas from the wells for which preincubation with 1.0-2.0 mg/ml ristocetin was performed, approximately 850 cpm were recorded.

Similar experiments were performed using preincubation (again in 50 μl volumes) of ^{125}I-vWF with botrocetin. Without botrocetin essentially no counts above background were

recorded. Using preincubation concentrations of botrocetin of about 0.5 μg/ml and above approximately 2100 cpm per well were recorded. Bound radioactivity rose sharply to near 2000 cpm/well between 0 and approximately 0.25 μg/ml of preincubating botrocetin.

What is claimed is:

1. A peptide which inhibits binding of von Willebrand factor to platelet membrane glycoprotein 1b and/or glycoprotein 1b expressed on the surface of any cell of megakaryocytic lineage selected from the group of peptides consisting of:

NLDRCELTKLQVDGT
QVDGTLPVLGTLDLS
TLDLSHNQLQSLPLL
QTLPALTVLDVSFNR
LKTLPPGLLTPTPKL
NCEILYFRRWLQDNA
QDNAENVYVWKQGVD
SNVASVQCDNSDKFP

2. A peptide of Claim 1 having the amino acid sequence NLDRCELTKLQVDGT and constituting residues 61-75 of the amino terminal end of glycocalicin.

3. A peptide of Claim 1 having the amino acid sequence QVDGTLPVLGTLDLS and constituting residues 71-85 of the amino terminal end of glycocalicin.

4. A peptide of Claim 1 having the amino acid sequence TLDLSHNQLQSLPLL and constituting residues 81-95 of the amino terminal end of glycocalicin.

5. A peptide of Claim 1 having the amino acid sequence QTLPALTVLOVSFNR and constituting residues 97-111 of the amino terminal end of glycocalicin.

6. A peptide of Claim 1 having the amino acid sequence LKTLPPGLLTPTPKL and constituting residues 136-150 of the amino terminal end of glycocalicin.

7. A peptide of Claim 1 having the amino acid sequence NCEILYFRRWLQDNA and constituting residues 210-224 of the amino terminal end of glycocalicin.

8. A peptide of Claim 1 having the amino acid sequence QDNAENVYVWKQGVD and constituting residues 221-235 of the amino terminal end of glycocalicin.

9. A peptide of Claim 1 having the amino acid sequence SNVASVQCDNSDKFP and constituting residues 241-255 of the amino terminal end of glycocalicin.

10. A peptide comprising any sequential subset of the amino acid sequence of a peptide of Claim 1 and derivatives thereof and which inhibits binding of von Willebrand factor to platelet membrane glycoprotein 1b and/or glycoprotein 1b expressed on the surface of any cell of megakaryocytic lineage.

11. A derivative of a peptide according to Claim 1 or Claim 10 which includes an additional peptide sequence.

12. A method for inhibiting activation or aggregation of platelets and/or adhesion of platelets to surfaces comprising contacting the platelets with a peptide according to Claim 1, 10 or 11 in an amount effective to inhibit said activation, aggregation, or adhesion.

13. A method for inhibiting thrombosis in a patient which comprises administering to said patient a peptide according to Claim 1, 10 or 11 in an amount effective to inhibit said thrombosis.

14. A composition comprising 2 or more peptides of Claim 1.

15. A derivative of a peptide according to Claim 1 or 11 in esterified, acetylated or glycosylated form.

16. A polymer which inhibits binding of von Willebrand factor to platelet membrane glycoprotein Ib and/or glycoprotein Ib expressed on the surface of any cell of megakaryocytic lineage and which includes the following multiple domains:

domain A - a series of amino acids which constitutes any subset of the amino acid sequence of the 45 kDa amino terminal fragment of GPIbα;

domain B - a series of amino acids which constitutes any subset of the amino acid sequence of the 45 kDa amino terminal fragment of GPIbα and

which may be the same or different from that of domain A; and

domain C - a linker which joins domain A and domain B.

17. A polymer according to Claim 16 wherein said series of domain A and B are the same.

18. A polymer according to Claim 16 wherein said series of domain A and B are different.

19. A polymer according to Claim 16 wherein said linker comprises a monomeric or polymeric group.

20. A polymer according to Claim 19 wherein said linker comprises a sequence of amino acids.

21. A polymer according to Claim 16 wherein domain A comprises QVDGTLPVLGTLDLS or TLDLSHNQLQSLPLL and domain B comprises SDKFPVYKYPGKGCPTLGDEGDTDLYDYY.

22. A polymer according to Claim 16 wherein domain A comprises any subset of the amino acid sequence of QVDGTLPVLGTLDLS or TLDLSHNQLQSLPLL and domain B comprises any subset of the amino acid sequence of SDKFPVYKYPGKGCPTLGDEGDTDLYDYY.

23. A synthetic polymer which inhibits binding of von Willebrand factor to platelet membrane glycoprotein Ib and/or glycoprotein Ib expressed on the surface of any cell of megakaryocytic lineage and which comprises one or more sequences of amino acids of the glycoprotein Ibα chain, said sequence(s) being normally positioned at or near the surface of the Ibα chain in its native conformation and capable of interacting with von Willebrand factor.

24. A method for inhibiting activation or aggregation of platelets and/or adhesion of platelets to surfaces comprising contacting the platelets with a derivative of Claim 15 in an amount effective to inhibit said activation, aggregation, or adhesion.

25. A method for inhibiting thrombosis in a patient which comprises administering to said patient a derivative of Claim 15 in an amount effective to inhibit thrombosis.

26. A method for inhibiting activation or aggregation of platelets and/or adhesion of platelets to surfaces comprising contacting the platelets with a polymer of Claim 16 in an amount effective to inhibit said activation, aggregation, or adhesion.

27. A method for inhibiting activation or aggregation of platelets and/or adhesion of platelets to surfaces comprising contacting the platelets with a polymer of Claim 23 in an amount effective to inhibit said activation, aggregation, or adhesion.

28. A method for inhibiting thrombosis in a patient which comprises administering to said patient a polymer of Claim 16 in an amount effective to inhibit thrombosis.

29. A method for inhibiting thrombosis in a patient which comprises administering to said patient a polymer of Claim 23 in an amount effective to inhibit thrombosis.

30. A method for inhibiting activation or aggregation of platelets and/or adhesion of platelets to surfaces comprising contacting the platelets with a Ser^{251}-Tyr^{279} fragment of glycoprotein Ibα.

31. A method for inhibiting thrombosis in a patient which comprises administering to said patient a Ser^{251}-Tyr^{279} fragment of glycoprotein Ibα.

32. A polymer according to Claim 16 wherein domain B comprises a peptide chosen from one of SDKFPVYKYPGKGCPTLGDEGDTDLYDYY, SDKFPVYKYPGKGCP, GKGCPTLGDEGDTDL, GDTDLYDYYPEEDTE, EEDTE and EEDTEGDKVRATRTV.

33. A polymer according to Claim 16 wherein domain A comprises a peptide chosen from one of QVDGTLPVLGTLDLS, TLDLSHNQLQSLPLL, QTLPALTVLOVSFNR, and NLDRCELTKLQVDGT.

34. A $pCDM8^{neo}$-based expression plasmid having the identifying characteristics of pMW1 or pMW2.

35. A host cell transformed by a plasmid according to Claim 34.

36. A recombinant DNA expression plasmid or viral expression vector encoding a polypeptide which inhibits binding of von Willebrand factor to platelet membrane glycoprotein Ib, said plasmid or vector including a nucleotide sequence encoding the amino acid sequence from approximately His^{1} to approximately Ala^{302}, inclusive, of the amino terminal region of platelet membrane glycoprotein Ibα, or one or more sequential subsets thereof.

37. An expression plasmid or viral expression vector according to Claim 36 wherein said nucleotide sequence encodes the amino acid sequence from approximately His^{1} to approximately Thr^{294}.

38. An expression plasmid or viral expression vector according to Claim 36 wherein said nucleotide sequence encodes the amino acid sequence from approximately Gly^{271} to approximately Glu^{285}.

39. An expression plasmid or viral expression vector according to Claim 38 wherein said nucleotide sequence encodes also the amino acid sequence from approximately Gln^{71} to Ser^{85}.

40. An expression plasmid or viral expression vector according to Claim 36 wherein said nucleotide sequence encodes the amino acid sequence from approximately Ser^{251} to approximately Tyr^{279}.

41. An expression plasmid or viral expression vector according to Claim 36 wherein said nucleotide sequence further encodes a signal peptide.

42. An expression plasmid according to Claim 36 which is derived from pCDM8, $pCDM8^{neo}$, pcDNA1, $pcDNA1^{neo}$, pMAM, $pMAM^{neo}$ or Rc/CMV.

43. A mammalian host cell transformed by an expression plasmid or viral expression vector according to Claim 36.

44. A host cell according to Claim 43 which is capable of expressing and secreting a polypeptide comprising essentially the 45 kDa tryptic fragment of glycocalicin.

45. A process for producing a polypeptide having the biological activity of the 45 kDa tryptic fragment of glycocalicin comprising:

providing a stable, extrachromosomally replicable plasmid or viral expression vector capable of directing in mammalian cells the expression of a nucleotide sequence encoding an amino acid sequence which includes said 45 kDa fragment, said nucleotide sequence further encoding as part of said amino acid sequence amino acids which are not native to said 45 kDa fragment, and which are oriented at the carboxy terminus of said 45 kDa fragment;

transforming said mammalian cells with said plasmid or viral expression vector; and

maintaining said transformed mammalian cells under conditions permitting the expression of said polypeptide.

46. A process according to Claim 45 wherein said plasmid is selected from the group consisting of pMW1 or pMW2.

47. A process according to Claim 45 wherein said nucleotide sequence encodes the entire His^{1}-Leu^{610} glycoprotein Ibα polypeptide.

48. A process according to Claim 45 further comprising the step of recovering said polypeptide.

49. An expression plasmid or viral expression vector according to Claim 40 wherein said nucleotide sequence encodes also the amino acid sequence from Gln^{71} to Ser^{85}.

50. A viral expression vector according to claim 36 which is based upon a retrovirus or a baculovirus.

51. A DNA sequence encoding the fragment of glycoprotein Ibα consisting essentially of the sequence of amino acids from approximately residue His^{1} to approximately residue Ala^{302} or one or more sequential subsets thereof.

52. An expression plasmid or viral expression vector capable of directing the expression of a polypeptide, and which contains a transcriptional promoter followed downstream by a DNA sequence according to Claim 51.

53. An expression plasmid or viral expression vector according to Claim 52 containing a signal peptide-encoding sequence positioned upstream from and in proper reading frame with said glycoprotein Ibα-encoding DNA sequence, said signal sequence capable of directing or facilitating the secretion of the polypeptide from a eucaryotic cell.

54. A recombinant eucaryotic or procaryotic host cell transformed with an expression plasmid or viral expression vector according to Claim 52.

55. A process for producing a biologically active polypeptide from DNA corresponding to that fragment of mature glycoprotein Ibα comprising the amino acid sequence from approximately residue His^{1} to approximately residue Ala^{302} thereof, or a subfragment thereof, comprising:

(A) constructing a DNA sequence, a first region of which encodes said fragment or subfragment and a second region of which encodes a signal peptide, said second region positioned upstream from and in proper reading frame to said first region;

(B) inserting said DNA sequence into a suitable plasmid or vector to create a construct comprising an expression plasmid or viral expression vector, said construct being capable of directing the expression in, and secretion from, eucaryotic cells of said fragment or subfragment;

(C) transforming a eucaryotic host cell with said expression plasmid or viral expression vector; and

(D) maintaining said transformed host cell under conditions resulting in expression within the host cell and secretion therefrom of the fragment or subfragment, said conditions resulting also in the

fragment or subfragment assuming a tertiary structure which is recognized by one or more conformation-dependent glycoprotein Ibα-specific antibodies.

56. A process according to Claim 55 in which there is effected glycosylation of the fragment or subfragment.

57. A biologically active polypeptide comprising essentially a His^{1}-Thr^{294} or His^{1}-Ala^{302} fragment of glycoprotein Ibα, or one or more sequential subsets thereof, said polypeptide produced by a process which utilizes cloning of a recombinant DNA molecule.

58. A polypeptide according to Claim 57 having domains of tertiary structure exhibited by platelet glycoprotein Ibα.

59. A process according to Claim 55 in which the fragment or subfragment is assembled into a biologically active structure absent the simultaneous expression of glycoprotein Ibβ or glycoprotein IX.

60. A process according to Claim 55 in which the host cell recognizes the glycoprotein Ibα fragment or subfragment as a protein which should be processed for secretion.

61. A therapeutic composition comprising one or more of the polypeptide structures according to Claim 57 or 58 effective to inhibit binding of von Willebrand factor to platelets, and a pharmaceutically acceptable carrier.

62. A method of inhibiting platelet activation and/or aggregation which comprises contacting platelets with an effective amount of a composition according to Claim 61.

63. A method of inhibiting adhesion of platelets to surfaces which comprises contacting platelets with an effective amount of a composition according to Claim 61.

64. A method of inhibiting thrombosis in a patient which comprises administering to such patient an effective amount of a composition according to Claim 61.

65. An antibody which is specific for glycoprotein Ibα or a polypeptide comprising one or more sequential subsets

thereof, said antibody being made by a process of immunizing animals with a polypeptide according to Claim 57 or 58 and then isolating the specified antibodies generated thereby.

66. A process for expressing the full length GPIb(α) polypeptide, His^{1}-Leu^{610}, or a fragment thereof, comprising:

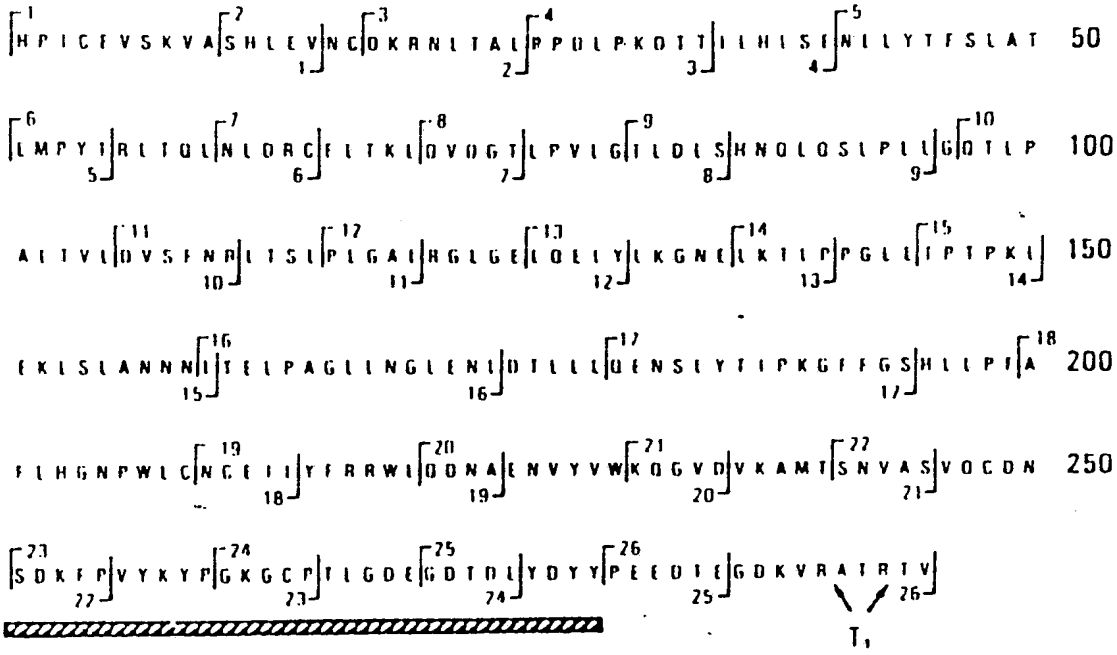
(A) constructing a DNA sequence which encodes said full length polypeptide or fragment;

(B) inserting said DNA sequence into a suitable plasmid or vector to create a construct comprising an expression plasmid or viral expression vector, said construct being capable of directing the expression in cells of said full length polypeptide or fragment;

(C) transforming a host cell with said expression plasmid or viral expression vector; and

(D) maintaining said transformed host cell under conditions resulting in expression of the full length polypeptide or fragment.

67. A process according to Claim 66 in which the full length polypeptide, His^{1}-Leu^{610}, or a fragment thereof, is expressed absent the simultaneous expression of glycoprotein Ibβ or glycoprotein IX.


HPICEVSKVASHLEVNCDKRNLTALPPDLPKDTTIHISENLLYTFSLAT 50
LMPYIRLTQLNLDRCELTKLQVDGTLPVLGTLDLSHNQLQSLPLLGQTLP 100
ALTVLDVSFNRLTSLPLGALRGLGELQELYLKGNELKTLPPGLLTPTPKL 150
EKLSLANNNITELPAGLLNGLENLDTLLLQENSLYTIPKGFFGSHLLPFA 200
FLHGNPWLCNCEILYFRRWLDDNAENVYVWKQGVDVKAMTSNVASVQCDN 250
SDKFPVYKYPGKGCPTLGDEGDTDLYDYYPEEDIEGDKVRATRTV
T1

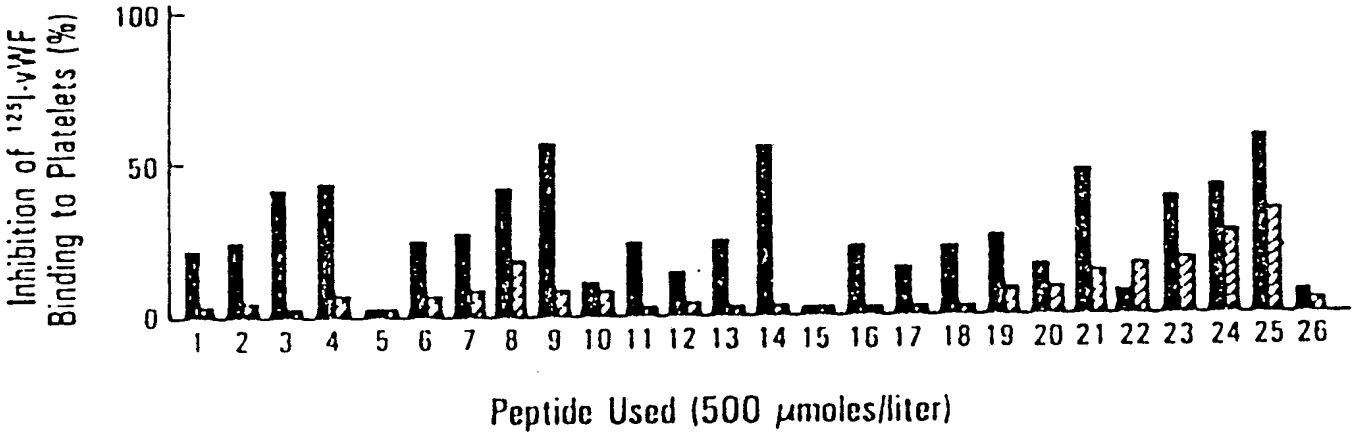

Inhibition of 125I-vWF Binding to Platelets (%)
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
Peptide Used (500 μmoles/liter)

FIGURE 1

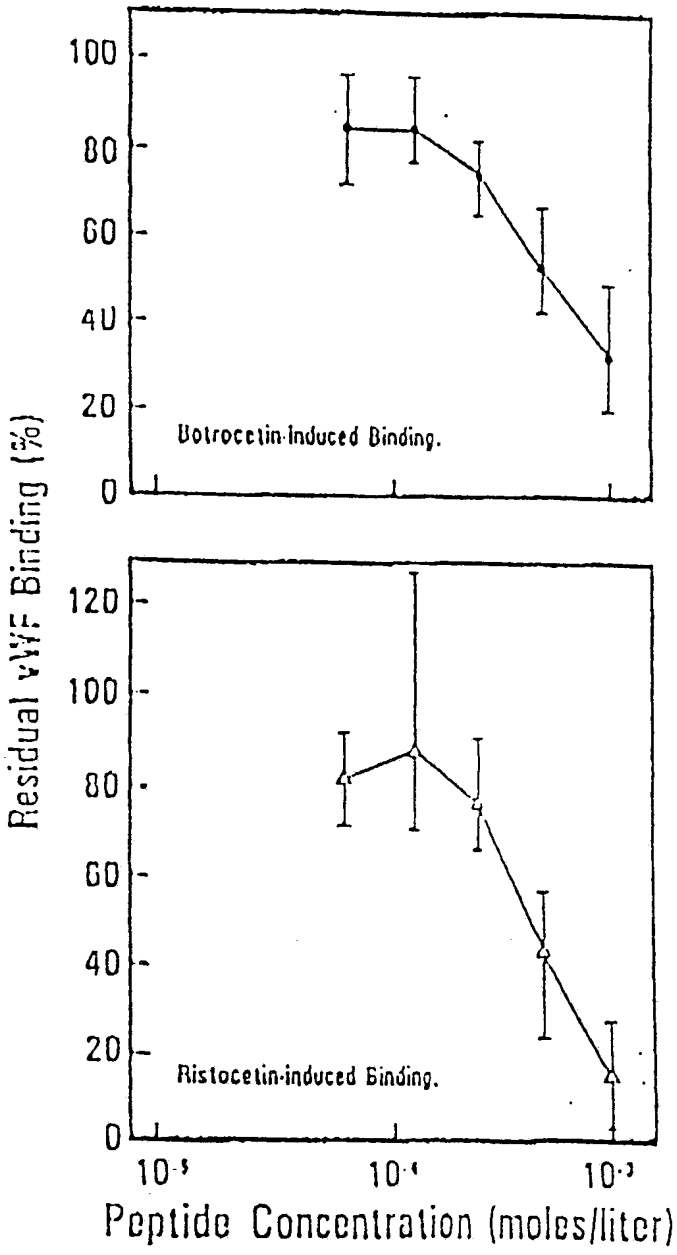

100
80
60
40
20
0
Botrocetin-induced Binding.
120
100
80
60
40
20
0
Ristocetin-induced Binding.
Residual vWF Binding (%)
10^{-5}
10^{-4}
10^{-3}
Peptide Concentration (moles/liter)

FIGURE 2

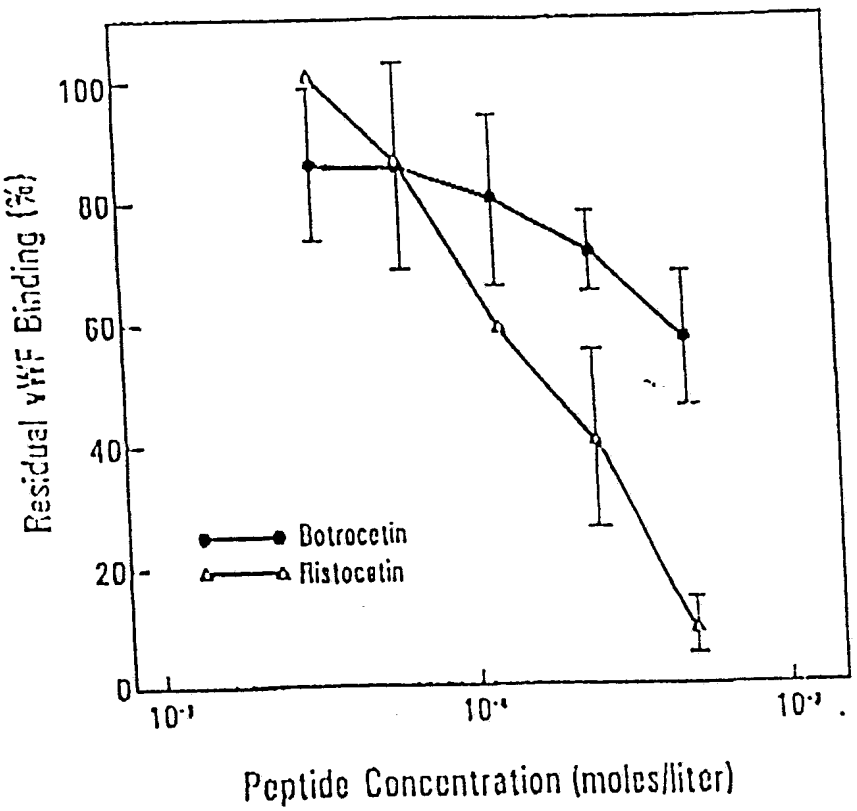

100
80
60
40
20
0
Botrocetin
Ristocetin
Peptide Concentration (moles/liter)

FIGURE 3

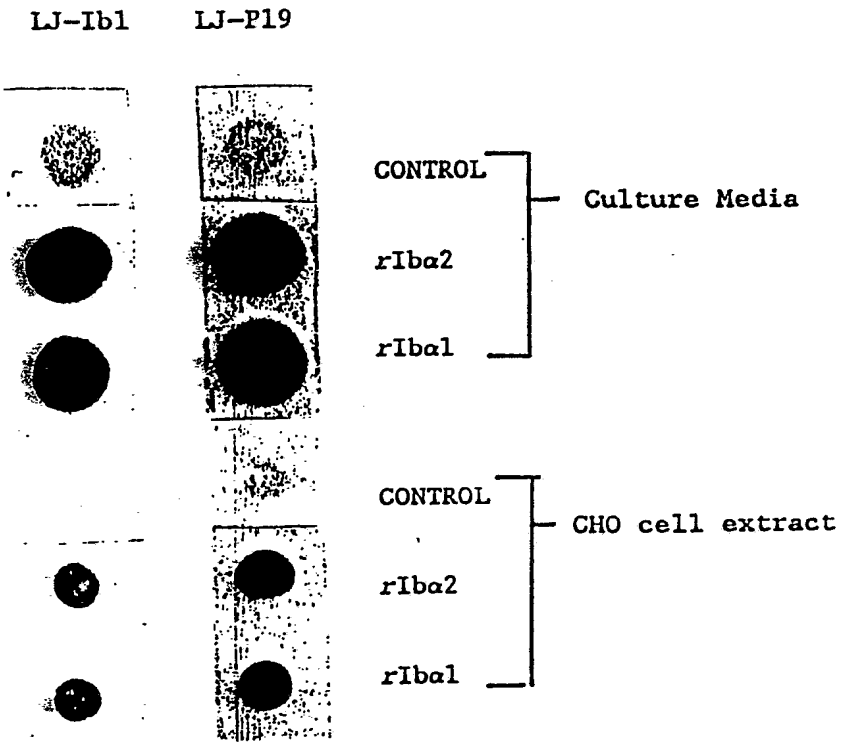

LJ-Ib1
LJ-P19
CONTROL
rIbα2
rIbα1
Culture Media
CONTROL
rIbα2
rIbα1
CHO cell extract

FIGURE 4

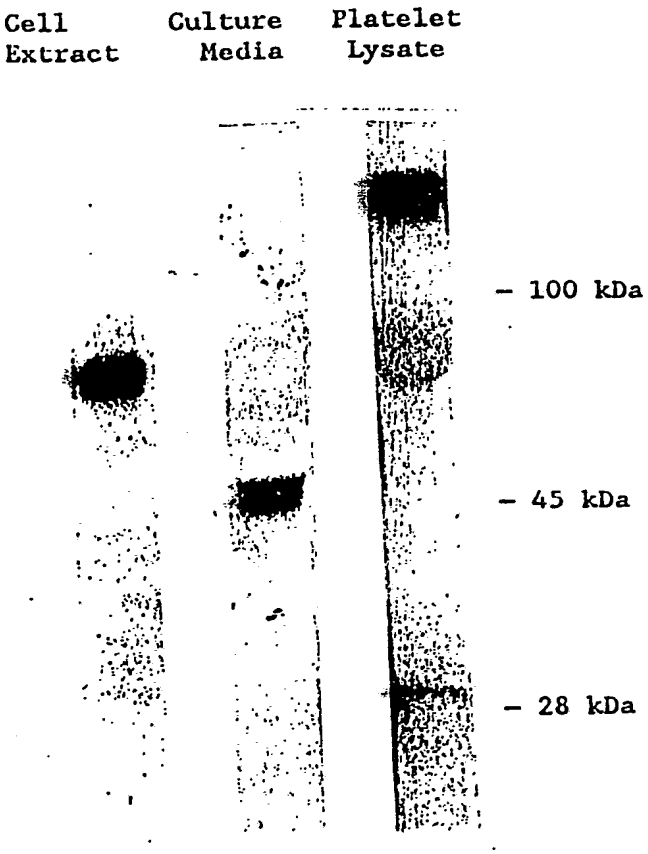

Cell Extract
Culture Media
Platelet Lysate
– 100 kDa
– 45 kDa
– 28 kDa

FIGURE 5

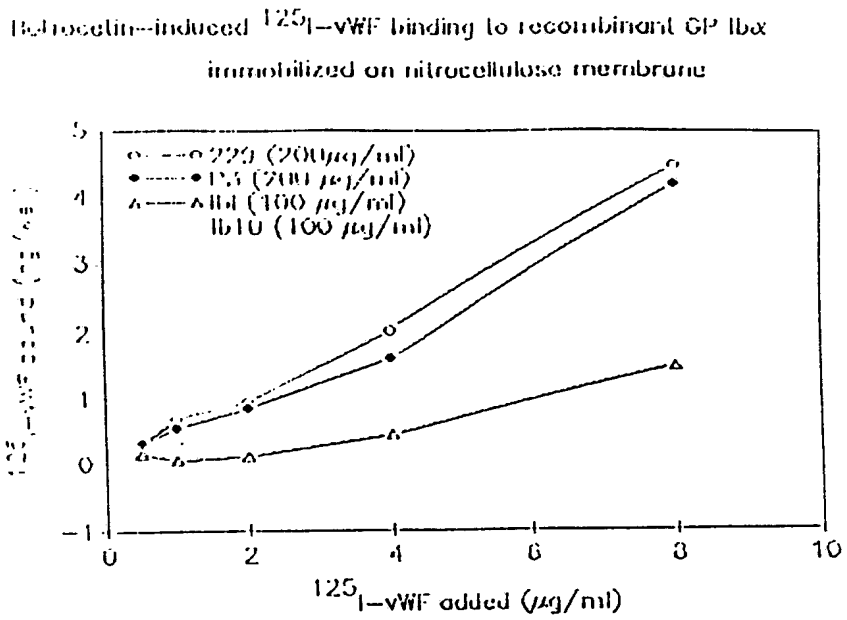

Botrocetin-induced 125I-vWF binding to recombinant GP Ibα
immobilized on nitrocellulose membrane
229 (200μg/ml)
(200 μg/ml)
(100 μg/ml)
Ib10 (100 μg/ml)
5
4
3
2
1
0
−1
0
2
4
6
8
10
125I-vWF added (μg/ml)

FIGURE 6

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/00087

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 37/02; C07K 3/00, 3/02, 7/08, 13/00, 15/06, 15/14

US.CL.: 530/324,325,326,350,381,382,383,395; 514/8,12,13,14,435/320.1, 240.1,240.2,70.1

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S. CL.:	435/70.1,240.1, 240.2, 320.1; 514/12,13,14,8; 530/324,325,326,350,381,382,383,395

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	The Journal of Biological Chemistry, Vol.261, No.27, issued 25 September 1986, Handa et al., "The von Willebrand Factor-binding Domain of Platelet Membrane Glycoprotein Ib, pages 12579-12585, see abstract.	1-2,12-13,16,18-29 32-33,26,41-46,48, 50-64
Y	The Journal of Biological Chemistry, Vol.253, No.10, issued 25 May 1978, Okumura et al., "Platelet Glycocalicin: Interaction with Thrombin and role as Thrombin Receptor of the Platelet Surface," pages 3435-3443, see abstract.	1-2,12-13,16,18-29 32-33,36,41-46,48, 50-64
Y	Biochimica et Biophysica Acta, Vol. 729, issued 1983, Solum et al., "Demonstration of a New Glycoprotein Ib- Related component in Platelet Extracts Prepared In The Presence of Leupeptin", pages 53061, see abstract	1-2,12-13,16,18-29 32-33,36,41-46,48, 50-64
Y	Biochemistry, Vol.22, No.23, issued 1983, Carnahan et al. "Comparative Analysis of Glycopeptides Derived from Human Platelet Membrane Glycoprotein Ib," pages 5384-5389, see abstract.	1-2,12-13,16,18-29, 32-33,36,41-46,48, 50-64

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 MAY 1991	06 JUN 1991

International Searching Authority	Signature of Authorized Officer
ISA/US	LESTER L. LEE

Form PCT/ISA/210 (second sheet) (July 1989)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	J. Clinical Invest., Vol.77, issued March 1986, Bockenstedt et al., "Structural Basis of von Willebrand Factor Binding to Platelet Glycoprotein Ib and Collagen", pages 743-749, see abstract	1-2,12-13,16,18-29, 32-33,36,41-46,48, 50-64
Y	Journal of Biological Chemistry, Vol.261, No.1, issued 05 January 1986, Fujimura et al., "A Reduced and Alkylated 52/48-kDa fragment Beginning at Amino Acid Residue 449 Contains The Domain Interacting with Platelet Glycoprotein Ib", pages 381-385, see abstract.	1-2,12-13,16,18-29 32-33,36,41-46,48, 50-64

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . . . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [3]

This International Searching Authority found multiple inventions in this international application as follows:

I. That group comprising a peptide and method of using, classified in class 514 (claims 1-13) and each specie of claim 1.

II. The group comprising the composition of a mixture of peptides classified in class 514, subclass 12 (claim 14) (See attachment)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
1-2,12-13,16,18-29,32-33,36,41-46,48,50-64 as to the species paid for in the election. (see attachment)

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without the payment of any additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	US,A, 4,703,039 (Hawiger et al.) 27 October 1987, See entire document.	1-2,12-13,16,18-29, 32-33,36,41-46,48, 50-64
Y	US,A 4,661,471 (Hawiger et al.) 28 April 1987, See Summary of Invention.	1-2,12-13,16,18-29, 32-33,36,41-46,48,50-64
Y	US,A 4,666,884 (Hawiger et al.) 19 May 1987, See summary of Invention	1-2,12-13,16,18-29, 32-33,36,41-46,48, 50-64
A	US,A 4,683,291 (Zimmerman) 28 July 1987, see entire document.	1-2,12-13,16,18-29, 32-33,36,41-46,48, 50-64
Y	Proc. Natl. Acad. Sci., Vol.84, issued August 1987, Lopez et al., "Cloning of the x chain of human platelet glycoprotein Ib: A transmembrane protein with homology to leucine-rich x_2-glycoprotein, see abstract.	1-2, 12-13, 16, 18-29, 32-33, 36, 41-46, 48, 50-64
Y	Chemical Abstracts, Vol. 104, issued 1986, Michelson et al., "Partial Characterization of a binding site for von Willebrand Factor on Glycocalicin", see page 438, column 2, abstract no. 104:86015j.	1-2, 12-13, 16, 18-29, 32-33, 36, 41-46, 48, 50-64

Continuation of Part VI Observations where unity of invention is Lacking

III That group comprising the derivatives of peptide containing additional peptide seqeunces, Classified in Class 530, subclass 325, (claims 11 and 15) and each species of peptide included in the claims,

IV That group comprising the polymer and method of using the polymer classified in class 514, subclass 12, (claims 16-29,32-33) and each species of the polymer included in the claims,

V That group comprising the method, of inhibiting thrombosis in a patient using Ser^{251}-Tyr^{279}, classified in class 514, subclass 12, claims (30-31)

VI That group comprising the pCDM8-based plasmid, recombinant DNA, a mammalian host cell transform, a process for producing a polypeptide, a viral expression vector, a DNA sequence encoding fragment, A recombinant encaryotic or procaryotic host cell classified in class 435 subclass 320.1, (Claims 34-56,59-60) and the species of each product above,

VII That group comprising a biologically active polypeptide of His^{1}-Thr^{294} or His^{1}-Ala^{302}, classified in class 530, subclass 350 (claims 57-58 and 61-64) and the species of His^{1}-Thr^{294} or His^{1}-Ala^{302},

VIII That group comprising the antibody classified in class 530 subclass 387, (Claim 65).

IX That group comprising the process for expressing a polypeptide classified in class 435 subclass 240.2 (claims 66-67) and each specie of peptide included in the claims.

The inventions listed as Groups I-IX do not meet the requirements for Unity of Invention for the following reasons: the intermediate of Group I can be used to inhibit thrombosis as well as prepare the products of Groups II, III and IV, there is no correlation between the peptides of Group I and the method of Group V, the products and methods of Group VI, the active peptide of Group VII, the antibody of Group VIII or the process of expressing the polypeptide of Group IX. Each invention in Groups IV-IX are not dependent upon Group I for novelty and therefore there is a lack of any unity between the inventions of said Groups.

During a telephonic requirement for election, on 08 May 1991 applicant's representative, Mr. Alexis Barron, elected the invention of Groups IV and VII and the species of Group I-
NLDRCELTKLQVDGT
Group IV- A-QVDGTLPVLGTLDLS
B-SDKFPVYKYPGKGLPTLGDEGDTDLYDYY
Group VII His^{1}-Ala^{302} for examination.

Applicant's representative also authorized the charging to the Deposit Account for payment of additional examination fees totaling $300 for the examination of said inventions. The additional examination fees have been charged to Deposit Account Number 19-5425.

Any inquiry concerning this communication should be directed to Lester L. Lee at telephone number (703) 308-3997.